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## Corticotropin Releasing Activity of a Pepsin Labile Factor in the Hypothalamus.* (24149)

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Acid extracts of calf hypothalamus elevate the level of blood ACTH in the decerebrate rat(1). The possibility was entertained that these extracts contained a corticotropin releasing factor (CRF), *i.e.*, a factor which travels from the hypothalamus *via* the portal venous system to the adenohypophysis to release ACTH. The present study was designed to evaluate the activity of hypothalamic extracts in a more specific test object, namely, the median eminence lesioned rat(2) and to determine whether the activity could be accounted for by the presence of ACTH and vasopressin. The results indicate that a third and as yet unidentified factor is in part responsible for ability of the hypothalamic extracts to induce adrenal ascorbic acid depletion in the median eminence lesioned rat.

*Methods.* Calf brains were dissected at the slaughter house. The pituitary stalk was transected immediately dorsal to the diaphragm sella. The median eminence area with attached pituitary stalk was separated from the rest of the hypothalamus, placed on dry ice and transported to the laboratory. Each piece of tissue has been designated a stalk median eminence unit (SME unit). The frozen tissue was placed in a Waring blendor which contained 2 ml of 0.2 M acetic acid per SME unit added, usually 20 SME units in 40

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TABLE I. Adrenal Ascorbic Acid Depletion in Acute Median Eminence (ME) Lesioned Rats Following ACTH, Pitressin or Stalk-Median Eminence (SME) Extracts.

| Exp. No. | Material inj. | Dose in fraction of SME unit | Pressor activity, mu | Adrenal ascorbic depletion, mg/100 g | |
|---|---|---|---|---|---|
| | | | | Hypox. | ME lesioned |
| 1 | Acid saline or polymixin B | | | | 18 ± 13*(13)† |
| 2 | a. ACTH, .5 mu | | | 58 ± 6*(20)† | 50 ± 12 (13) |
| | b. " , 2.0 " | | | 139 ± 5 (14) | 150 ± 7 (13) |
| 3 | a. Pitressin | | 5,000 | 77 ± 11 ( 9) | 172 ± 8 ( 7) |
| | b. " | | 500 | | 144 ± 11 ( 5) |
| | c. " | | 100 | 5 ± 13 ( 5) | 90 ± 15 ( 8) |
| | d. " | | 20 | | 14 ± 8 ( 9) |
| 4 | a. SME extract, A-23 | .4 | 670 (560 to 800)‡ | 201 ± 15 ( 6) | 203 ± 12 ( 5) |
| | b. *Idem* | .025 | 42 ( 35 to 50)‡ | 39 ± 12 (11) | 117 ± 6 (15) |
| 5 | a. SME extract, M-7 oxyc. eluate | .1 | 100§ | | |
| | b. SME extract, M-7 oxyc. supernatant | .1 | 10§ | 26 ± 10 (11) | 148 ± 14 ( 8) |
| | c. SME extract, M-29 oxyc. eluate | .1 | 80§ | 204 ± 17 ( 6) | 191 ± 10 ( 9) |
| | d. SME extract, M-29 oxyc. supernatant | .1 | 12§ | 61 ± 14 ( 5) | 154 ± 10 (15) |
| 6 | a. SME extract, M-29 oxyc. supernatant + pepsin | .1 | | | 42 ± 7 ( 6) |
| | b. SME extract, A-23 | .025 | 42 ( 35 to 50)‡ | 39 ± 12 (11) | 117 ± 6 (15) |
| | c. *Idem* + pepsin | " | 46 ( 34 to 63)‡ | 31 ± 10 ( 6) | 49 ± 9 (11) |
| | d. *Idem* + ACTH, 1.0 mu | " | | 141 ± 14 ( 5) | |
| | e. *Idem* + pepsin | " | | 171 ± 7 ( 5) | |

* Mean and stand. error. † No. of animals. ‡ Potency with 95% fiducial limits.
§ Estimate based on 2 or more responses each of standard and of unknown.

ml of 0.2 M acetic acid. The mixture was homogenized at full speed for 120 seconds and immersed in a 100°C water bath, refluxed for 10 minutes, and centrifuged for one hour at 16,000 r.p.m. in a preparative head (SW-25.1) of a Spinco ultracentrifuge. The clear aqueous phase (SME extract) was decanted and stored at 4°C; activity was undiminished after 4 weeks of storage. Washed oxycellulose(3) in a quantity of 250 mg per 10 SME units was added to the crude extract, the pH adjusted to 3.0 with glacial acetic acid and the mixture stirred intermittently for 12 hours. The oxycellulose was filtered on a sintered glass funnel. The fraction unadsorbed by oxycellulose was designated the SME oxycellulose supernatant and the material eluted from the oxycellulose with 0.1 M HCl (2 times with 10 ml) designated the SME oxycellulose eluate. In the pepsin experiments, 4 ml of a solution which contained NaCl (0.9%), HCl (0.01 M) and USP pepsin (5 μg/ml) were incubated for 2 hours at 37°C with 1 ml of SME extract which contained the equivalent of 0.5 SME unit. Crude extracts, oxycellulose fractions and pepsin treated extracts were appropriately diluted for bioassay with a solution which contained NaCl (0.9%) and HCl (0.01 M). Doses in column 3 of Table I are expressed as fractions of an SME unit.

Assays for CRF activity were performed in the acute hypothalamic lesioned rat (250 to 300 g)(4). Immediately after lesioning the rats were placed in metabolic cages. Rats with 24-hour urine volumes greater than 75 ml and water consumptions greater than 110 ml were used for assay 48 hours after lesioning. Extracts were infused intravenously over 5 minutes following left adrenalectomy. Thirty minutes later the right adrenal was removed. The difference between concentrations of adrenal ascorbic acid in the left and right adrenals is an index of ACTH action during the period between removal of the two glands. The response may represent ACTH

release and/or ACTH injected. ACTH activity of the extracts was determined by assay in 24-hour hypophysectomized rats (110 to 135 g)(5). Vasopressin content was estimated by pressor response of rats pretreated with phenoxybenzamine (U.S.P. XV method).

*Results.* Acid saline alone or with addition of polymyxin B, up to and including toxic amounts, does not induce adrenal ascorbic acid depletion in the acute lesioned rat (Exp. 1, Table I). Lack of response is not attributable to hyposensitivity of the adrenal to ACTH; 0.5 and 2.0 milliunits (mu) ACTH evoked adrenal ascorbic acid depletion of a magnitude similar to that observed in the 24-hour hypophysectomized rat (Exp. 2). Significant depletion was observed with quantities of Pitressin as low as 100 milliunits (mu) of pressor activity (Exp. 3). The observed response was not entirely due to release of pituitary ACTH as Pitressin also induced adrenal ascorbic acid depletion in hypophysectomized rats. The greater response in presence of the pituitary may be interpreted as indicating a direct effect of Pitressin on the adenohypophysis. McCann's claim(6) of direct action of vasopressin on the adenohypophysis is confirmed.

An acetic acid extract (SME, A-23), prepared from calf stalk-median eminence tissue depletes adrenal ascorbic acid in the lesioned assay animals. At a dose of 0.4 of an SME unit, degree of adrenal ascorbic acid depletion was equal and near maximum in lesioned and in hypophysectomized rats (Exp. 4a). However, at a dose of 0.025 SME unit, response was much greater in the lesioned than in the hypophysectomized animal (Exp. 4b). This suggests that there is an insufficient quantity of ACTH in the extracts to account for the activity in the lesioned rat. 0.025 SME unit contained 42 mu of pressor activity, a quantity which acting alone is insufficient to account for the adrenal ascorbic acid depletion in the lesioned rat. The possibility is not ruled out that small quantities of ACTH acting in conjunction with vasopressin potentiate (7) to yield the result observed in the lesioned rat. CRF activity was retained by the extracts after adsorption of most of the ACTH-like and vasopressin-like activity on oxycellulose. Approximately 90% of the pressor activity of extract M-7 was adsorbed on oxycellulose (Exp. 5a and 5b). The pressor activity of SME, M-7, oxycellulose supernatant was insufficient to account for the adrenal ascorbic acid depletion observed in the lesioned rat (cf. Exp. 3d with Exp. 5b). The quantity of ACTH in the oxycellulose supernatant was barely detectable. Adsorption of a second extract (SME, M-29) on oxycellulose eliminated approximately 85% of the pressor activity from the supernatant and led to a striking reduction of response in the hypophysectomized rat (Exp. 5c and 5d). CRF activity was not proportionately reduced. Again, the possibility that a small amount of ACTH plus a small amount of vasopressin induced a response in the lesioned rat which represented potentiation cannot be ruled out.

More definitive evidence for presence of a factor in SME, distinct from ACTH and from vasopressin, which induces adrenal ascorbic acid depletion in the lesioned rat, was derived from experiments with pepsin. The factor largely responsible for adrenal ascorbic acid depletion in the lesioned rat was pepsin labile (Exp. 5d, cf. 6a; 6b, cf. 6c), whereas inherent pressor activity and activity of added ACTH were unaffected by pepsin (Exp. 6b, c, d, e). Incubation of SME, A-23 in absence of pepsin resulted in no significant change in activity in lesioned rats.

*Discussion.* Ablation of the median eminence area of the hypothalamus results in failure of ACTH release in response to a variety of otherwise effective stimuli(4) (ether anesthesia plus unilateral adrenalectomy, histamine, epinephrine, toxic doses of polymyxin B). Heretofore, only 2 agents, vasopressin and ACTH, have been demonstrated to induce adrenal ascorbic acid depletion in the lesioned rat(4). The present study demonstrates that extracts prepared from tissue composed of calf pituitary stalk and median eminence area are also effective. The extracts contain both vasopressin-like activity and ACTH-like activity. Assays of diluted extracts and of oxycellulose fractions suggest that these 2 agents are only in part respon-

sible for the activity in the lesioned rat. Resistance of ACTH and of vasopressin to pepsin digestion has been previously reported and is confirmed in the present study(8,9). Loss of CRF activity of the SME extracts following incubation with pepsin constitutes definitive evidence for a CRF distinct from vasopressin.

Pitressin (Exp. 3), purified natural vasopressin and synthetic vasopressin(7) have been demonstrated to deplete adrenal ascorbic acid in the hypophysectomized rat. The pepsin labile factor may also have an extrapituitary action. The fact that Pitressin and the pepsin labile factor induce a much greater degree of adrenal ascorbic acid depletion in the lesioned than in the hypophysectomized rat suggests that the major action of both agents is on the adenohypophysis.

A working concept for action of vasopressin in inducing adrenal ascorbic acid depletion has been presented(7). Vasopressin may release ACTH from the adenohypophysis, from the kidney, and possibly from other tissues by competing for polypeptide binding sites. The pepsin labile factor may act in a similar fashion. That the release of ACTH is relatively specific is indicated by failure of oxytocin(6), glucagon (unpublished observation) and polymyxin B to deplete adrenal ascorbic acid in the lesioned rat.

Both the pepsin labile factor and vasopressin satisfy the classical criteria for endocrine roles. They are present in an area whose ablation results in an endocrine deficiency (failure of ACTH release in response to acute stimuli) and administration of either agent to the lesioned rat corrects the deficiency (release of ACTH). Demonstration of the pepsin labile factor at site of origin of the portal venous system lends support to the concept that a substance elaborated in the hypothalamus is carried *via a* vascular link to the adenohypophysis where it initiates ACTH release.

*Summary.* Ability of acid extracts of calf hypothalamus (stalk-median eminence area) to induce adrenal ascorbic acid depletion in the median eminence lesioned rat is attributable in large measure to a pepsin labile factor distinct from ACTH and from vasopressin.

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## Species Differences in Cholesterol Biosynthesis by Arterial Tissue.* (24150)

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The role of the artery in production or maintenance of atherosclerosis is unknown. Since the artery itself is the primary target of the disease, its own metabolic activity may be of great importance in formation of atheroma. Until recently the evidence available(1,2,3) seemed to indicate that the lipids found in atheromata are deposited directly from the plasma. However, studies with $P^{32}$ indicate that at least part of the phospholipid moiety

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is actually synthesized by arterial tissue *in situ*(4) and that this synthetic rate can be influenced by naturally occurring substances such as epinephrine(5) and cortisone(6). Reports utilizing radioactive tracer technics have indicated that aortas of rabbits(7,8), fowl(7,9), swine(10), and calves(11) are capable of incorporating $C^{14}$-labeled acetate into cholesterol. In only the latter 2 species, however, was the purification of isolated cholesterol adequately performed to eliminate all radioactive contaminants. Since cholesterol is the major sterol in atheromata, the incorporation of $C^{14}$-labeled acetate into cholesterol by arterial tissue is of interest. This process was studied in several different species of animals for the following reasons: 1) There is a well known variation of arterial response in different species to feeding high cholesterol diets. 2) Differences have been reported for content of various enzymes in coronary artery as compared to aorta and in atheromatous as compared to non-atheromatous areas of the coronary artery(12). 3) To date adequate cholesterol purification procedures in studies of arterial cholesterol biosynthesis were performed in only 2 species of animals. 4) The possibility of inhibiting arterial cholesterol synthesis, if there be such, by vanadium salts or other inhibitors of cholesterol biosynthesis has attractive therapeutic implications.

*Materials and methods.* Human aorta was obtained at autopsy within 8 hours of death or during great vessel surgery. It was immediately placed in iced buffer and taken to the laboratory where adherent fat was stripped from the adventitial surface. Two 0.5-1.0 mm thick slices were made from the intimal side with a Stadie-Riggs microtome. Approximately 3 g of tissue were placed in 20 ml phosphate buffer(13) containing 10 mg sodium acetate-1-$C^{14}$ (0.5 mc/mM) and incubated for 2 hours at 37°C in an atmosphere of 95% oxygen and 5% $CO_2$. At the end of incubation period carrier cholesterol was added as indicated (Tables I and II). The entire contents of the incubation flask were made to a concentration of 70% ethanol and 5% potassium hydroxide and saponified under reflux for 2 hours. The non-saponifiable material was extracted with petroleum ether, (B.P. 30-60°C). The combined petroleum ether extracts were evaporated to dryness under a stream of nitrogen and the residue taken up in 90% ethanol. Cholesterol was precipitated from this solution as the digitonide. Cholesterol digitonide was washed with 90% ethanol, 40% ethanol – 60% ether, and ether successively. Radioactivity of the digitonide was determined and the cholesterol then purified *via* dibromide(10). The dibromocholesterol was repeatedly recrystallized from methanol to constant radioactivity. Radioactivity was determined using a thin end-window Geiger-Mueller tube. A sufficient number of counts were obtained so that 2 counts above background were reliably higher than ambient ionization counts at a level of confidence less than 0.01. When coronary artery was obtained, the easily accessible portions (usually proximal portion of right and left coronary arteries) were rapidly dissected free from adherent fat, opened along their long axes and incubated without preparing slices. Groups of the various species of adult animals were sacrificed by a blow on the head and the aorta from the aortic ring to the level of renal artery removed and chilled in cold buffer. The adventitial surface was stripped free of adherent fat and a pool of arteries (Table II) from the same species and sex, was incubated in a manner similar to that described above for coronary artery. Because of the relative thickness of dog aorta, slices of this tissue were prepared for incubation.

*Results.* Although human aorta and coronary artery are capable of incorporating acetate into a digitonin-precipitable substance (Table I), this material is not cholesterol, inasmuch as all radioactivity disappears from the cholesterol fraction with preparation of the dibromide. Synthesis of a digitonin-precipitable substance which was not cholesterol was also found by Schwenk and Stevens (14) when they incubated Yoshida ascites tumor cells with radioactive acetate. Increasing the incubation time to 5 hours, or addition to the incubating medium of di and triphosphopyridine nucleotides, adenosine triphos-

TABLE I. Incorporation of Acetate-1-$C^{14}$ into Cholesterol by Arterial Tissue (Humans).

| Aorta | Age | Sex | Cholesterol carrier, mg | Radioactivity* Cholesterol carbon | | Cause of death |
|---|---|---|---|---|---|---|
| | | | | Digitonide | Dibromide | |
| J.H. | 50 | ♀ | 0 | 608 | 0 | Obtained during surgery for insertion of Hufnagel valve |
| W.D. | 15 | ♂ | 4 | 1690 | 0 | *Idem* |
| T.R. (area mostly atheromata) | 48 | ♂ | 0 | 229 | 0 | Trauma |
| T.R. (area relatively free of atheromata) | | | 0 | 368 | 0 | " |
| H.S. | 54 | ♀ | 0 | 204 | 0 | Acute leukemia |
| E.S. | 34 | ♂ | 2 | 195 | 0 | *Idem* |
| A.M. | 78 | ♂ | 0 | 201 | 0 | Carcinoma of rectum |
| J.S. | 25 | ♂ | 0 | 233 | 0 | Rheumatic heart disease |
| W.T. | 58 | ♂ | 0 | 404 | 0 | Carcinoma of prostate (rec'd estrogen therapy) |
| C.R. | 6 mo | ♀ | 4 | 1870 | 0 | Suffocation |
| *Coronary artery* | | | | | | |
| E.S. | 34 | ♂ | 8 | 100 | 0 | Acute leukemia |
| A.C. | 51 | ♀ | 4 | 140 | 0 | Chronic myelogenous leukemia |

* Radioactivity = counts/min. at infinite thickness × mg cholesterol carrier.

phate, or glucose singly or in combination, also did not induce conversion to cholesterol. The possibility exists that conversion of acetate to cholesterol does not take place when autopsy material is used because of inactivation of a necessary enzyme following death. However, the fact that aorta obtained during surgery behaved in a similar manner mitigates against an enzyme inactivation as the explanation. Also it has been shown by Maier and Haimovici(15) that at least the oxidative capacity of dog and rabbit aorta does not change appreciably when tissue from freshly sacrificed animals is compared to that from animals kept under conditions simulating those of humans from the time of death until autopsies are performed. The apparent inability of human aorta and coronary artery to incorporate acetate into cholesterol adds further corroboratory evidence that cholesterol found in atheroma in humans is not synthesized *in situ* but rather is deposited from plasma cholesterol.

A similar inability to incorporate acetate into cholesterol is seen in the aortic tissue of dogs, cats, and rats (Table II). This is in agreement with studies of Gould(16) on dog aorta. Although the possibility exists that addition of carrier cholesterol could dilute out a minimal amount of radioactivity, it seems unlikely. The data in Table II, however, are in disagreement with the results of

TABLE II. Studies on Incorporation of Acetate-1-$C^{14}$ into Cholesterol by Arterial Tissue (Experimental Animals).

| Aorta | Sex | Carrier cholesterol, mg | No. of animals pooled | Radioactivity* Cholesterol carbon Digitonide | Dibromide |
|---|---|---|---|---|---|
| Dog | ♀ | 0 | 1 | 590 | 0 |
| | ♀ | 2 | 1 | 620 | 0 |
| | ♂ | 2 | 1 | 216 | 0 |
| Chicken | ♂ | 4 | 4 | 632 | 440 |
| | ♀ | 4 | 4 | 1840 | 829 |
| Rabbit | ♂ | 4 | 3 | 1150 | 576 |
| | ♀ | 4 | 3 | 1400 | 936 |
| Guinea pig | ♂ | 8 | 7 | 600 | 257 |
| | ♂ | 4 | 7 | 450 | 226 |
| | ♀ | 8 | 7 | 2280 | 514 |
| | ♀ | 4 | 7 | 926 | 732 |
| Rat | ♂ | 8 | 10 | 1480 | 0 |
| | ♀ | 8 | 10 | 1970 | 0 |
| Cat | ♀ | 4 | 4 | 316 | 0 |

* Radioactivity = counts/min. at infinite thickness × mg cholesterol carrier.

Ranney and Weiss(17), who report increase in rate of "cholesterol synthesis" of rat aorta following administration of α-p-biphenylyl butyric acid. Cholesterol in the investigations of Ranney and Weiss was assayed for radioactivity as the total non-saponifiable fraction. This fraction contains several other sterols in addition to cholesterol(10). The importance of employing cholesterol purification procedures when one wishes to measure "cholesterol synthesis" is further borne out by our unpublished findings that even plasma itself is capable of converting $C^{14}$-labeled acetate to a digitonin-precipitable substance which is not cholesterol. Further, experiments reported here were carried out *in vitro* to eliminate the possibility of extra-arterial cholesterol synthesis in the intact animal providing preformed labeled cholesterol which could then be deposited in the aorta.

From the literature and data in Tables I and II, various species of animals can be divided into 2 groups based upon aortic cholesterol metabolism. Group I comprises those animals whose aortas *can* incorporate acetate into cholesterol and includes rabbits, chickens, guinea pigs, swine(10), and calves(11). Group II comprises those animals whose aortas *cannot* incorporate acetate into cholesterol and would include humans, dogs, rats, and cats. Two other characteristics of the grouping of these species appear evident. Animals in Group I consume primarily a herbivorous type diet and can be made atherosclerotic with relative ease by feeding a high cholesterol diet while animals in Group II are carnivores and omnivores and it is difficult, if not impossible, to produce atherosclerosis in these animals by high cholesterol intake alone. At present the significance of this difference is not evident and no satisfactory explanation is available for the observed differences. Preliminary attempts to characterize the radioactive digitonin-precipitable substance synthesized by aortas of Group II animals have been unsuccessful.

*Summary.* Coronary arteries and aortas of humans and aortas of dogs, cats, and rats cannot incorporate $C^{14}$-labeled acetate into cholesterol *in vitro*. Aortas of guinea pigs, rabbits, and chickens can accomplish this conversion.

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## *In vitro* Assay of Hog Intrinsic Factor with Rat Liver Homogenates. (24151)

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In this paper we describe an *in vitro* assay for hog intrinsic factor which has proven to be very useful. The method is based upon earlier work of Miller(1) and others(2-4) in which they showed that intrinsic factor could increase the Vit. $B_{12}$ uptake of rat liver slices. In our method liver homogenates are used, since the results are similar to those with slices but a much greater precision can be obtained. Certain intrinsic factor preparations were found to cause a lower Vit. $B_{12}$ uptake than expected in this *in vitro* assay, indicating presence of an inhibitor. The relationship between this inhibitor and an inhibitory intrinsic factor similar to that reported by Tauber and co-authors(5) is under investigation.

*Materials and methods.* Male Sprague-Dawley rats were used which weighed 200-350 g. They had been fed a standard high-vitamin casein diet including 100 $\mu$g Vit. $B_{12}$/kg diet. Animals were decapitated and bled before hepatectomy. Liver homogenates (1 g liver/5 ml buffer) were prepared in loose-fitting Teflon and glass homogenizer in ice and were strained through cheesecloth. The buffer was at pH 7.1 and had the following composition in mM/liter: K, 135; Mg, 25; Ca, 12.5; Cl, 20.5; and $PO_4$, 5.3. The preservative-free $Co^{60}$-Vit. $B_{12}$ (Merck) was adjusted to approximately 90 m$\mu$g/ml in 0.14 M potassium chloride. A Dubnoff water bath was used for incubations which were generally 3½ hours at 37°C under air atmosphere. The same Vit. $B_{12}$ uptake was observed under air as under oxygen or nitrogen. Each 25 ml Erlenmeyer flask contained 1 ml of homogenate, 2 ml buffer, 0.2 ml $Co^{60}$-Vit. $B_{12}$ solution, and 1.4 ml of isotonic potassium chloride which contained varying amounts of IFC.* After incubation the contents of each flask were transferred to a small test tube and centrifuged. The sediment was resuspended once in 4 ml of isotonic potassium chloride and again centrifuged. Supernatant was poured and drained from washed sediment and the radioactivity of $Co^{60}$-Vit. $B_{12}$ bound in the sediment was determined in scintillation well.

*Results.* Fig. 1 shows rate of Vit. $B_{12}$ uptake by rat liver homogenates and IFC with varying amounts of Vit. $B_{12}$. In this experiment flasks containing 5 times the regular quantities of materials were incubated at 37°, and 3 ml aliquots were withdrawn at intervals into iced tubes and centrifuged immediately. The IFC, WIF-54B from hog pylorus, had activity of 1.7 USP units/mg as shown by this homogenate-Vit. $B_{12}$ uptake assay. When uptake in presence of IFC is corrected for control uptake by the homogenate, the curves in Fig. 2 are obtained. The action of IFC at 37° is so rapid that aliquots removed at zero-time showed considerable uptake; the process can be appreciably slowed by reducing the quantity of Vit. $B_{12}$ and temperature. Despite this rapid action, for laboratory convenience all subsequent experiments were incubated for 3½ hours. Enhancement of IF activity by calcium as reported by Herbert (2) with rat liver slices was confirmed as shown in Fig. 3. Calcium was always present in the standard assay at final concentration of 8.2 mM/liter as indicated by arrow in the Figure.

Activities of IFC from hog pylorus were proportional to the initial uptake slope, m$\mu$g Vit. $B_{12}$/mg. IFC, as determined by a plot of Vit. $B_{12}$ uptake *vs.* $\mu$g IFC. Typical plots are shown in Fig. 4. Depression of uptake after the peak may be caused by IF itself and not by an inhibitor (although see below), for when plots of IFC varying from 0.017-2.0 USP units/mg are adjusted to superimpose the ascending slopes, the descending slopes are often very similar. To correct for variation among rat livers, a standard IFC, 315-W9 having 0.2 unit/mg, was included in each set of assays, and all activities were calculated relative to

* The following abbreviations are used: IF, intrinsic factor; IFC, intrinsic factor concentrate. All IFC prepared from hog tissues.

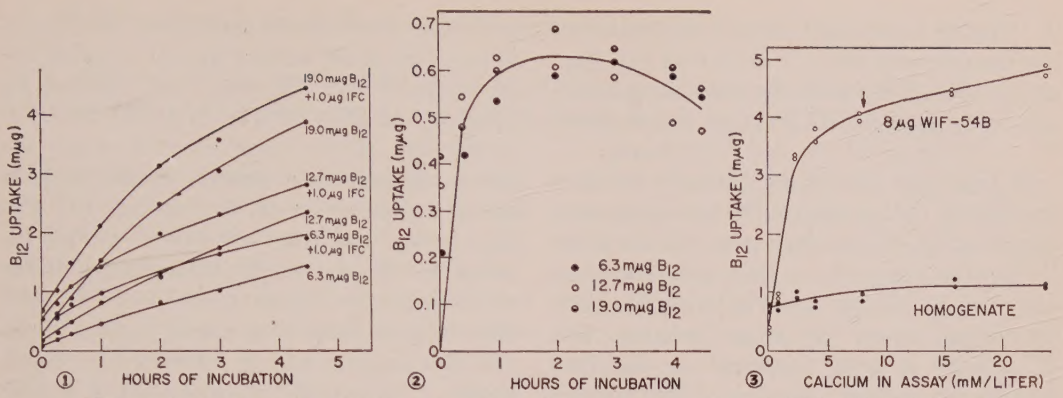
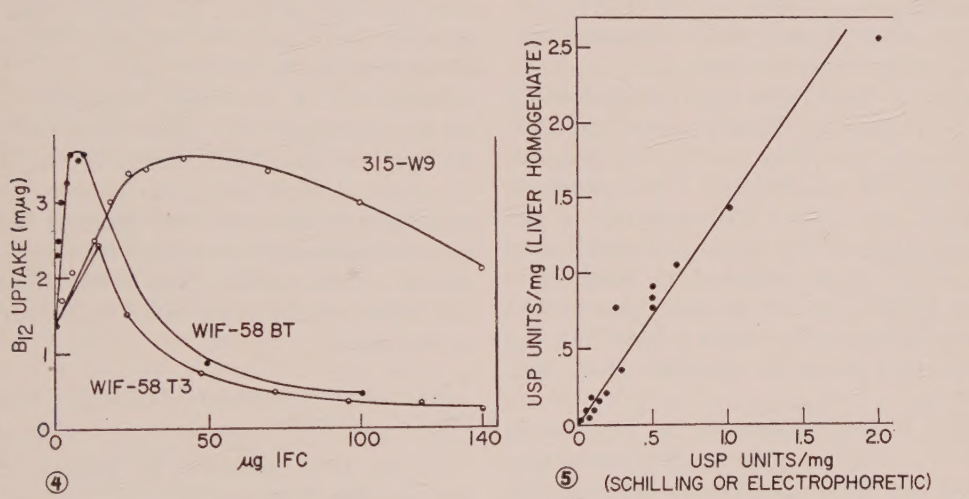



FIG. 1. Rate of vit. $B_{12}$ uptake in presence of liver homogenate and IFC WIF-54B. Quantities are per 3 ml aliquots.

FIG. 2. Rate of vit. $B_{12}$ uptake in presence of IFC WIF-54B (1.0 μg) after correction for uptake by homogenate.

FIG. 3. Uptake of vit. $B_{12}$ by rat liver homogenate and WIF-54B (8 μg) as a function of $Ca^{++}$ concentration. $Ca^{++}$ concentration used in standard assays is shown by arrow.



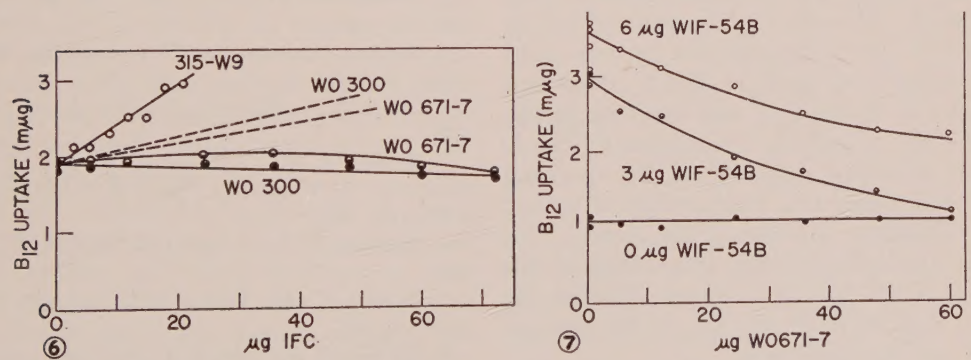




FIG. 4. Uptake of vit. $B_{12}$ by varying amounts of 3 hog pyloric IFC.

FIG. 5. Comparative assays of 15 IFC from hog pylorus.

FIG. 6. Vit. $B_{12}$ uptake by varying amounts of 2 hog duodenal IFC. Broken lines indicate uptakes expected from their Schilling potencies.

FIG. 7. Inhibition of WIF-54B, a hog pyloric IFC, by increasing quantities of WO671-7, a hog duodenal IFC.

it. Uptake by 315-W9 with 15 different liver homogenates was 98 ± 3 SEM mμg Vit. $B_{12}$/mg 315-W9. The uptake by these same homogenates without added IFC was less uniform, 2.1 ± 0.2 mμg Vit. $B_{12}$/ml hogomenate.

In Fig. 5 are plotted the potencies of many hog pyloric IFC as determined by homogenate uptake of Vit. $B_{12}$ *vs.* their potencies by either the Schilling assay(6) or electrophoretic assay described by Barlow and Frederick(7). The correlation among the assays indicates that IF activity is being measured by the liver homogenate method. The line of least squares in Fig. 5 does not coincide with the expected 45° line for a 1:1 relationship between the assay methods. This is due, perhaps, to an error in ascribing a potency of 0.2 unit/mg to the reference standard 315-W9. The randomness of the points is due to combined variability of the 3 assay methods.

By use of this *in vitro* assay an inhibitor of Vit. $B_{12}$ uptake in IFC from hog duodenum was detected as shown in Fig. 6. Preparations WO-300 and WO-671-7 had potencies of 0.067 and 0.050 USP unit/mg in the Schilling assay. The uptakes calculated from these potencies are indicated by broken lines in the Figure. As can be seen, these typical duodenal preparations cause a lower Vit. $B_{12}$ uptake than would be expected from their potencies.

There is also evidence for an inhibitor in some IFC from hog pylorus. In these preparations the initial uptakes agreed with the Schilling assay but maximum uptakes were depressed. This effect is demonstrated in Fig. 4 by WIF-58T3. An inhibitory fraction could be isolated from such an IFC.† Inhibitory samples of duodenal IF also inhibited uptake of Vit. $B_{12}$ by pyloric IF as shown in Fig. 7. When increasing amounts of the duodenal IFC were added to a fixed amount of pyloric IFC, inhibition is shown by the curves. The same duodenal inhibitor thus inhibited the action of IFC from both tissues. Whether the effect is on IF itself, Vit. $B_{12}$, or the homogenate was not established.

†O. Walasek, unpublished.

Further experiments with livers and gastric juices from other animal species revealed extreme specificity. This was also reported by Johnson and Driscoll(8). With rat liver homogenates, gastric juices from 4 other rats and 2 non-pernicious anemia human subjects either depressed or did not elevate the Vit. $B_{12}$ uptake. IFC from hog pylorus did not increase Vit. $B_{12}$ uptake by homogenates of livers from 8 mice, 2 hogs, 1 rabbit and 1 chick. This lack of interaction among various livers and IFs may be due to inhibitors as shown above. Herbert(3) has discussed a similar phenomenon.

It is evident that the liver homogenate assay, as described here, is a precise and convenient method for determination of hog pyloric intrinsic factor. The results obtained with this assay correlate very well with those of the Schilling technic in man.

*Summary.* A convenient laboratory assay for hog pyloric intrinsic factor using $Co^{60}$-Vit. $B_{12}$ and rat liver homogenates is described. By means of this *in vitro* assay the presence of an inhibitory substance was detected in certain crude preparations derived from hog duodenum. These showed a diminished response relative to activity in the Schilling urinary excretion assay.

Acknowledgements are made to Jean Loach for her technical assistance.

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# Hippocampal Lesions Following Administration of 3-Acetylpyridine. (24152)

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In testing hypotheses regarding the role of the hippocampus in emotion, memory, and learning, it would be desirable to observe the effects of complete removal of this structure (1). With usual technics, this is impossible to accomplish without causing extensive damage to other structures. In reporting acute experiments with 3-acetylpyridine (3AP), Hicks stated that cerebral changes were limited to destruction of cells in the hippocampus and supraoptic nuclei(2). Although the extent of damage in the hippocampus was not specified, this suggested to us the possibility of obtaining hippocampal lesions in chronic preparations which could be used for behavioral and neuro-anatomical studies. The findings in the mouse reported here have encouraged an extension of this investigation to cats and monkeys.

*Materials and methods.* Adult, male, noninbred albino mice and rats, maintained on stock diet, were used. The $LD_{50}$ of 3AP was 300-350 mg/kg for mice and 80 mg/kg for rats. A single intraperitoneal injection of $LD_{50}$ dose was given in water. Animals were sacrificed with ether and perfused through the heart with 10% formalin. The brains were mounted in celloidin and sectioned at 25 $\mu$. Every tenth section was stained with cresyl-violet for cells, and, in most instances, facing sections were stained by the method of Weil or Woelcke for myelin. For control, brains of 2 normal mice were prepared in the same manner. In a few instances the frozen section technic was used. The brains of 20 mice and 10 rats were examined.

*Results.* About 6 hours after injection of 3AP, rats developed an apparent weakness of extremities and rolled from side to side upon attempting to move. Surviving animals manifested inspiratory rhonchi, urinary incontinence, and weakness of hind legs. Gross pathological examination of organs and microscopic examination of brains were not remarkable.

*Mice.* Surviving mice lost weight and developed an unkempt appearance. Inspiratory rhonchi were characteristically present. About a third of the animals developed urinary incontinence and a high-stepping gait. Fiery red tongue was not noted. The animals were sacrificed at intervals from 4 hours to 2 months after treatment. The gross pathological examination was not remarkable. Histological examination was confined to the brain. Of the 20 mice examined, 15 showed slight to complete loss of neurons in areas of the hippocampus designated *CA3* and *CA4* (Fig. 1A) by Lorente de Nó(3) (area H3 of the Vogts). When the damage was slight, it was most conspicuous in the region of *CA3a*. In cases of moderate damage, there was an extension of cell loss, usually complete, to the rest of area *CA3* (Fig. 1C). With severe damage, there was a complete loss of neurons in *CA4* as well (Fig. 1D). There was no neuronophagia, and no increase in glia was evident. Of the 12 mice allowed to survive at least one month, 5 showed moderate to severe damage of the foregoing areas. In some cases, damage was irregularly present in other areas of the hippocampus and in the dentate gyrus, but in no other parts of the brain.

*Discussion.* The present study adds to the growing evidence that the metabolism of hippocampus is distinctive from that of other types of cortex. It is possible that our findings will eventually be found to have some correlation with the observations: (i) 3AP, which is an analogue of nicotinic acid, may act as a competitive antagonist of this substance(4); (ii) chronic nutritional deficiency in man, characterized by lack of niacin (pellagra), may be accompanied by psychotic manifestations; (iii) dithizone, a chelating agent which gives a reddish color when combined with zinc, stains and sharply demarcates the same area of the hippocampus destroyed by 3AP(5,6); (iv) *in vitro* studies suggest that the active site of DPN dependent

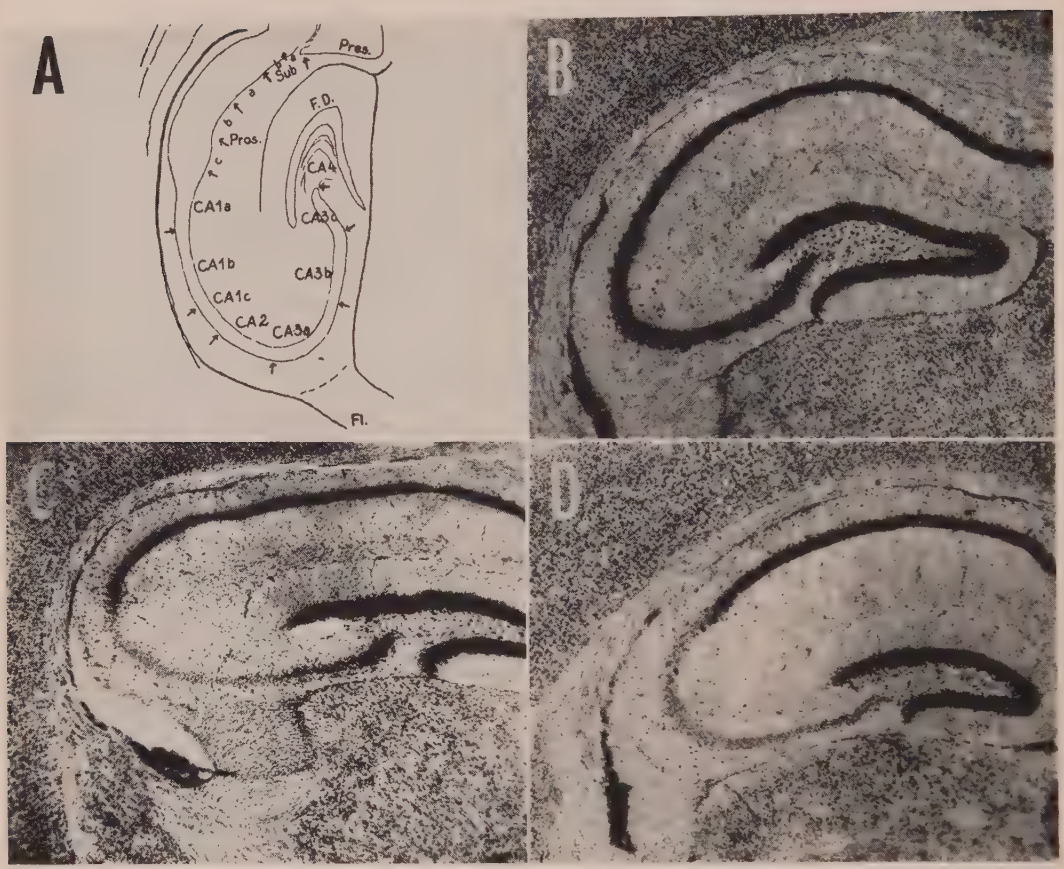


FIG. 1. A: Lorente de Nó's areal subdivision of hippocampus in mouse (Lorente de Nó, 1934); CA, cornu ammonis; F.D., fascia dentata; Pros., prosubiculum; Sub, subiculum; Pres., presubiculum. B: Nissl stain of section through "normal" hippocampus of mouse. C: Loss of neurons in region corresponding to *CA3* in 3AP-treated mouse. D: Section from another experiment showing, in addition, destruction in region of *CA4*.

systems may involve an interaction involving sulfhydryl groups and zinc(7); (v) administration of $C^{14}$-labeled isoniazid leads to high radioactivity in the hippocampus(8); (vi) isoniazid is related to iproniazid which inhibits one of the enzymes that participate in destruction of serotonin, noradrenalin, and other amines; (vii) serotonin is found in high concentration in the hippocampus(9); and (viii) radioautographic studies employing $S^{35}$-labeled l-methionine suggest a high protein turnover in the hippocampus(10).

*Summary.* Administration of 3-acetylpyridine (3AP) to non-inbred, adult mice results in cerebral changes restricted almost exclusively to areas *CA3* and *CA4* of the hippocampus. Ability to induce such lesions in chronic preparations has encouraged extension of this investigation to cats and monkeys with the eventual purpose of performing behavioral studies concerned with elucidating the functions of the hippocampus.

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## Properties of Myxoma Virus Transforming Agent. (24153)

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Investigations of the phenomenon described by Berry and Dedrick(1) have developed currently in several stages, the first having been to transform fibroma into myxoma virus in a predictable fashion. This has been accomplished in tissue culture as previously described(2,3). The present report concerns the nature of the heat-inactivated transforming agent (TAM) used in above experiments and of conditions under which it operates in tissue culture. The effect of enzymes, organic solvents, and the photodynamic action of light on this preparation were studied (Table I). Information gained by such means has proved of value in continuing studies to determine the relation of the nucleic acids of myxoma virus to the Berry-Dedrick phenomenon. The results are presented here.

*Materials and methods.* Transforming agent myxoma (TAM) was prepared by concentrating myxoma virus in an ultracentrifuge at 30,000 to 40,000 rpm in a No. 40 rotor, then heating the resuspended sediment at 65°C for 40 minutes. TAM mixed with live fibroma virus was inoculated into cultures of trypsinized rabbit kidney cells. Supernatant fluids harvested from these cultures 3 times a week, were tested for presence or absence of transformation by intracutaneous inoculation of rabbits. Since fibroma is a benign and myxoma a fatal disease of rabbits, these tests had a sharp end point. More complete details are given in previous publications(2,3). Additional methods are presented under various headings below.

*Results. Effect of organic solvents.* Infectivity of myxoma virus was destroyed when 1:5 suspensions were extracted with equal volumes of anaesthetic ether for 30 minutes to 2½ hours at room temperature. This was shown by separation and testing of the aqueous layer. No live virus was recovered from 7 lots of myxoma treated in this manner, and tested by intracutaneous inoculation of rabbits, as well as by passage in tissue culture. Two lots, 18 and 19, were further tested in rabbit skin in dilutions of $10^{-1}$ to $10^{-8}$. No live virus was revealed. The 7 lots of myxoma were now designated as ETHER-TAMS and when mixed with live fibroma virus, were tested for transforming activity in a series of tissue culture experiments. Table II shows that 4 of these ETHER-TAMS (Lots 18, 19, 28, and 39) were active in transformation, a finding indicated by appearance of live myxoma virus in 16 of 21 experiments. Aliquots from 2 of these ETHER-TAMS, were heated at 65° for 12 to 40 minutes. These exposures were well beyond those needed to inactivate live myxoma, as elsewhere described(3). The ETHER-TAMS subjected to both treatments in this manner appeared as effective as when ether was the only method used to inactivate.

TABLE I. Effect of Various Treatments on Myxoma Virus and on TAM (Transforming Agent Myxoma).

| Treatment | Activity of TAM | Infectivity of myxoma virus |
|---|---|---|
| Heat 65°C (3 to 45 min.) | Retained | Destroyed |
| Anesthetic ether | " | " |
| Chloroform | Destroyed | " |
| DNAse | Retained | ND* |
| Trypsin | " | ND |
| Visible light + toluidine blue | Destroyed | Not totally destroyed |
| Ultraviolet light | " | *Idem* |

* ND = Not done.

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† Cancer Institute, N.I.H.

TABLE II. Numbers of Positive Transformation Experiments Carried Out with Ether-Prepared TAMS, Shown in Relation to (1) Total Number of Experiments Performed, (2) Use of Heat in Addition to Ether Inactivation and (3) Possible Effect of Serum in Suspending Medium.

| Lot No. | Time at 65°C | Serum in preparation medium, % | Transformation in tissue culture, No. pos./total tested |
|---|---|---|---|
| 18 a | 12 min. | 10 | 5/5 |
| 18 b | none | " | 3/3 |
| 19 | 12 to 40 min. | " | 3/4 |
| 39 | none | 2 | 2/3 |
| 28 | " | none | 3/6 |
| 29 | " | " | 0/6 |
| 30 | " | " | 0/5 |
| 31 | " | " | 0/2 |

With ETHER-TAM 18, for example, 3 of 3 experiments were positive (*i.e.* showed transformation) with unheated material, and 5 of 5 other experiments were positive when the same material was heated for 12 minutes. Of 18 negative transformation experiments, 16 fell in a group of 4 ETHER-TAMS (28, 29, 30, 31, Table II) which had been prepared somewhat differently. In these lots, the original myxoma tumors had been suspended in buffered saline (pH 7.2) and no serum was used in subsequent processing. The most effective ETHER-TAMS (18 and 19, Table II) were suspended throughout preparation in Hanks' balanced salt solution containing 10% inactivated horse serum. Suspensions of myxoma virus exposed to chloroform, under conditions similar to those used for above ether extractions in which serum was present, showed no infectivity and no transforming activity in 15 tissue culture experiments.

*Enzyme treatment.* TAMS prepared by heating retained transforming activity after treatment both with *desoxyribonuclease* (DNase) and with trypsin. The TAMS were originally centrifuged and heated in media containing 10% calf serum, as described for previous experiments(3), but were given 2 additional centrifugations at 40,000 rpm to wash and resuspend them in buffered saline, pH 7.2. The pH was that of the nutrient tissue culture fluids. These TAMS were now exposed to 4-5 $\mu$g/ml of crystalline bovine pancreatic DNase (Worthington) for 1 to 2 hours at room temperature. Three different lots of TAM, including one previously treated with trypsin and one suspended in the original media with 10% calf serum, retained activity in the presence of DNase, as revealed by positive results in 9 experiments. Tests on aliquots of TAM suspensions showed that added DNA was rapidly hydrolyzed in the presence of the enzyme. Crystalline trypsin (Worthington), was allowed to act on one lot of TAM for 20 hours, in amounts of 0.8 mg/ml. The mixture was afterward heated at 65°C for an hour to destroy the enzyme. Six positive transformation experiments indicated that the activity of this single lot of TAM was unaffected by trypsin.

*Inactivation by photodynamic action.* A number of animal viruses have been shown to lose infectivity when illuminated with visible light in the presence of dissolved oxygen and a photodynamic dye(5,6). The reaction is a photosensitized oxidation of portions of the virus to which the dye is chemically bound. The mechanism of this reaction has not been reported in detail, but it is supposed that the viral nucleic acids provide the critical reaction sites. With these considerations in mind, 2 TAM preparations were irradiated with intense visible light after addition of toluidine blue O to a final concentration of 0.61 mg%. These preparations were found to have lost their ability to induce transformations, when they were tested subsequently in 6 tissue culture experiments. A control sample containing the dye and maintained in the dark, remained active as shown in 2 experiments.

*Inactivation by ultraviolet light.* Two experiments indicated that TAM exposed to ultraviolet light lost its activity under conditions which did not completely destroy the infectivity of live myxoma virus. The samples were irradiated for 10 minutes in a layer thickness of 2 mm with an incident intensity of 36 microwatts/cm$^2$ at 2537 Å.

*Sedimentation of TAM particles.* TAM prepared by heat-inactivation presumably consists of particles which are essentially of the same size as those of unheated myxoma virus, a finding suggested by the following experiment. Two lots of TAM were centrifuged

in a Spinco at 40,000 rpm for 1 hour. This centrifugation was the same as that used in the original preparation and transforming activity was recovered from the sediments, but not from the supernatant fluids.

*Fate of TAM in tissue cultures.* Simultaneous additions of TAM and live fibroma virus to rabbit kidney cultures led to transformations in nearly all experiments whereas addition of TAM alone, under similar circumstances, never resulted in appearance of any infectious virus(2,3). A number of questions arose from these experiments. First, how long might TAM survive in tissue cultures and second, would it attach to or enter cells in an absence of fibroma virus? Titrations of TAM were not attempted in experiments designed to answer these questions. A dosage of 0.45 ml of TAM, used for each 2 ounce bottle of tissue culture, possibly represented at least 3 times the amount required for transformation. In initial experiments, supernatant fluids were poured from 3 pairs of tissue culture bottles. TAM was then allowed to overlay the cell sheets directly for varying periods, ranging 5 to 30 minutes, after which an effort was made to wash it away with 3 changes of nutrient fluid. Fibroma virus was then added to the cultures. Transformations subsequently took place in the 15- and 30-minute experiments. No transformation took place after the 5-minute interval. The first of the wash fluids used in these 3 experiments were combined with live fibroma virus as a basis for 3 duplicate experiments, all of which were positive. There was thus evidence that TAM had been used originally in amounts exceeding those needed for transformation. The experiments were now repeated at intervals of 1 and of 24 hours before the addition of fibroma virus. Results were the same as at intervals of 15 and 30 minutes with the exception that wash fluids were not tested. It was concluded from this set of experiments that TAM could become bound to rabbit kidney cells by itself, and that it was not readily destroyed when incubated in such cultures at 36°C. A further question was whether TAM had to be added at the same time or before fibroma virus, or whether it could be added afterward. Two experiments were carried out for this purpose. TAM was added 24 hours and 48 hours respectively after fibroma virus, with transformations taking place within a week in both experiments. It has become evident that TAM can be effective whether added before, or as much as 48 hours after fibroma virus.

*Lack of immunizing capacity of TAM.* TAMS were tested routinely in rabbits and no sign of infection ever resulted, when a maximum of 2 ml of concentrated material was given by intracutaneous and intraperitoneal routes of inoculation(3). One wondered, however, whether such inoculations might not protect test animals against subsequent challenge with live myxoma virus. Seven rabbits were challenged accordingly, 12 to 14 days after inoculation. Of these animals, 3 different lots of ETHER-TAM had been tested in 3 of them and 4 different lots of heat-prepared TAM in 4. All of the animals challenged developed myxomatosis, in a minimum incubation time of 5 days. There was thus no evidence that TAM had any protective effect.

*Discussion.* The structure of myxoma virus and alterations resulting from TAM preparation are of interest to an analysis of the Berry-Dedrick phenomenon. Electron microscope studies indicate that myxoma has a relatively large protein coat surrounding an inner core of DNA, a structure which, as Farrant and Fenner(4) have shown, resembles that described for the related vaccinia virus (7). From another point of view, there is a basic structural difference between these 2 pox viruses. Andrewes and Horstman(8) found that fibroma and myxoma viruses were ether-sensitive while vaccinia was ether-resistant. The former viruses may have a soluble outer layer, possibly lipid in nature. One may speculate that heat and ether methods of preparing TAM both denature the protein coat, and that ether may also remove this outer lipid layer. Several things appear to be evident concerning the structure of the TAM particle. First, TAM particles sediment as readily in an ultracentrifuge as those of myxoma virus, indicating that their size has not

altered significantly. Second, it is probable that the central core of DNA remains intact. This core, lying within a coat of denatured protein, is relatively stable to storage and to moderate heating, and it is unaffected by exposure to DNase. It is, however, readily destroyed by visible light in the presence of toluidine blue and by ultraviolet irradiation.

TAM is seemingly inert when studied alone *in vivo* and *in vitro*. Inoculated rabbits not only fail to develop any signs of infection, but also fail to develop immunity to a subsequent challenge with live myxoma virus. A question of interest is what happens to TAM in the presence of live fibroma virus. A first thought was that the live virus might function in promoting entry of TAM into tissue culture cells. Once inside, TAM particles would lose their outer coats and the central DNA would replicate itself. The action of fibroma virus, however, is probably more specific. Several experiments indicated that TAM particles can attach to and possibly enter cells alone and even a related virus, vaccinia, will not lead to transformation when given simultaneously. Fibroma virus sets up special conditions after infecting a susceptible cell. Febvre *et al.*(9) have shown that all recognizable fibroma virus disappears shortly after entry and an area of viroplasm, the inclusion body, appears within the next 5 hours. Virus particles begin to emerge from this area about 8 hours after infection. One wonders whether TAM particles coming to this matrix area might not lose their outer coats and then replicate themselves from the central cores of DNA. Investigations are being continued to better characterize the transformation process and the interactions involved.

*Summary.* The transforming agent prepared from myxoma virus (TAM) is resistant to heat, to ether extraction, and to mild enzymatic digestion but is inactivated by the photodynamic action of toluidine blue. TAM particles can be sedimented by ultracentrifugation (Spinco). TAM becomes attached to rabbit kidney cells in 15 minutes, when inoculated by itself, is not destroyed by incubation in tissue cultures for 48 hours at 36°C, and can be added before or after live fibroma virus in transformation experiments. The transforming agent is not immunogenic against myxoma virus in rabbits.

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## On the Nature of Oxytocin and Vasopressin from Human Pituitary.* (24154)

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Comparison of pressor-antidiuretic hormones isolated in this laboratory from posterior pituitary glands of beef and hog has shown them to be cyclic octapeptide amides differing in one amino acid residue, the beef vasopressin containing arginine(1) and vasopressin from hog glands, lysine(2). The structure of arginine vasopressin, the cyclic disulfide of L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-arginylglycinamide, may be repre-

* This work was supported in part by grant from Nat. Heart Inst., P.H.S.

sented as follows: CyS-Tyr-Phe-Glu($NH_2$)-Asp($NH_2$)-CyS-Pro-Arg-Gly($NH_2$). In lysine vasopressin, the lysine residue occurs in the place occupied by arginine in arginine vasopressin. On the other hand, oxytocins from these 2 sources appear to be identical and differ from vasopressins in having an isoleucine residue in place of phenylalanine and a leucine residue in place of arginine or lysine(3). Thus, interest was aroused in establishing the nature of the posterior pituitary hormones, particularly the vasopressins, from glands of other species. Van Dyke, Engel and Adamsons(4) recently noted a difference between pharmacological effects of lysine and arginine vasopressin, namely that the ratio of pressor potency to antidiuretic potency was 1:1 for arginine vasopressin, and 6:1 for lysine vasopressin when the antidiuretic potency was determined by intravenous injection in the hydrated, unanesthetized dog. They also tested for these relative potencies in extracts of pituitaries of man, monkey, dog, rat, sheep, and camel, and found a 1:1 ratio to exist between pressor and antidiuretic potencies. These results suggested that arginine vasopressin was the pressor-antidiuretic hormone in these species, although, as van Dyke and his collaborators pointed out, their conclusions were tentative and must be confirmed by isolation of the hormone from each species and determination of the amino acid composition of each vasopressin. Recently we reported a procedure for isolation of oxytocin and vasopressin from posterior pituitary glands of beef and hog which was applicable to small scale work(5). In this method the hormones are separated as a complex attached to a protein fraction and subsequently separated from proteins and then from one another in high yield. The method has now been adapted to the study of oxytocin and vasopressin from the human pituitary.

*Methods.* Human pituitary lobes were placed in acetone at the time of post-mortem examination, and later the posterior lobes were separated and pulverized to a powder.† Preparations of the powder varied in oxytocic and pressor activity from 0.2 to 0.35 unit/mg. Oxytocic activity was measured by the avian depressor method of Coon(6), and the rat pressor assay was used to determine vasopressor activity(7). A preparation starting with 3.3 g of human posterior pituitary powder provided a sufficient quantity of active components for their subsequent characterization. This amount of acetone-desiccated powder was extracted twice with 100 ml of 0.25% acetic acid, then with 50 ml of acid (at −5°C with stirring). The extraction times were 17, 8, and 18 hours, respectively. The combined extracts were lyophilized, then dissolved in 0.25% acetic acid to give a concentration of about 30 units/ml. Sodium chloride was added to final concentration of 10% and the protein precipitate was removed by centrifugation. The protein precipitate resulting from addition of sodium chloride was suspended in a minimum volume of water and dialyzed against water. The contents of the sack were acidified and centrifuged to remove a small amount of sediment and the solution was lyophilized. The residue was dissolved in 0.25% acetic acid and the proteins precipitated by addition of TCA to a final concentration of 10%. The precipitate was dissolved in 0.25% acetic acid and again precipitated with TCA. The last step was repeated and the combined supernatants were added directly to a column (0.9 x 14.5 cm) of IRC-50 ($H^+$), which was washed with 0.25% acetic acid until the pH of the effluent was the same as that of the acid. It was then washed with water. Trichloracetic acid was removed by the washes leaving peptides bound to the resin. The oxytocic and vasopressor activities were separated by gradient elution chromatography(8) with ammonium acetate buffers, 0.1 M, pH 5.0 → 0.5 M, pH 7.7 → 0.75 M, pH 7.7 (Fig. 1). The fractions were desalted on IRC-50 ($H^+$) columns and the eluted components were lyophilized. Yields and degree of purification obtained at different stages are presented in Table I. It will be noted that the hormones of the human pituitary could be purified in a manner similar to that described

† The authors wish to thank Dr. Peter G. Condliffe, Nat. Inst. of Arthritis and Metabolic Diseases, N.I.H. for the posterior lobes of human pituitary used.

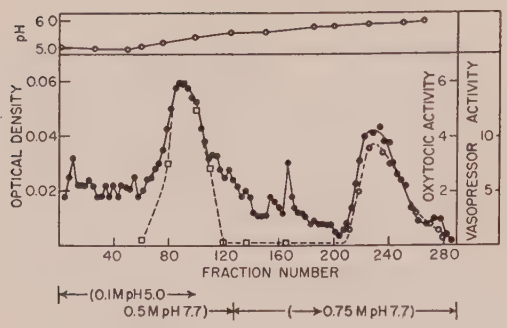


FIG. 1. Column separation of oxytocic and pressor principles. Resin, IRC-50; column, 0.9 × 14.5 cm; buffer, ammonium acetate; vol/tube, 1 ml; flow rate, about 2 ml/hr; optical density is of the Folin color on contents of every third tube. □, oxytocic activity; ○, pressor activity.

for beef and hog glands(5). Addition of 10% sodium chloride to the pituitary extract resulted in precipitation of a protein fraction containing the major part of the activity, although a greater part of the activity was soluble in this case than was true in the earlier work. However, the yield of the protein fraction was sufficient to permit further studies.

*Results.* Chromatographic behavior of the biologically active components of human posterior pituitary gland can be compared to previous results obtained for oxytocin and vasopressin derived from beef glands. In this system oxytocin is eluted at a pH of 5.3, and arginine vasopressin emerges at pH 6.0(5). The properties of human hormones on the column are those of oxytocin and arginine vasopressin. The agreement of the curve for the Folin color with that for biological activities was very good, indicating that the components were of high purity, particularly the vasopressin. No peptide material was still adsorbed on the column since Folin-positive material was not eluted after the column was washed with 4% ammonium hydroxide.

It should be pointed out that the yield of chromatographically separated components obtained from 3.3 g of acetone-dried pituitary glands amounted to 250 to 300 units of each activity. Since the effluent fractions from the chromatographic separation were analyzed by the Folin reagent, one-third of the activity was consumed in this process. The weight of material isolated after the column procedure represented a fraction of a milligram. Consequently, further work could only be of a qualitative nature for identification of the active components.

TABLE I. Purification of Oxytocin and Vasopressin from Human Posterior Pituitary Glands.

| Step | Total activity (units) V* | O* | Folin color, % |
|---|---|---|---|
| Extraction of pituitary powder with acetic acid followed by lyophilization | 974 | 683 | 100 |
| Precipitation of protein complex followed by dialysis | 414 | 369 | 31 |
| Dissociation of complex and gradient elution chromatography | 330 | 185 | † |
| Desalting and lyophilization | 174 | 68 | † |

* V = pressor activity; O = avian depressor activity.
† Not determined.

TABLE II. Comparison of Human Oxytocic Component with Oxytocin from Beef Glands. Paper electrophoresis on Whatman 3MM, 22 in long × 4 in wide. Mobility in mm indicates direction of movement. Paper chromatography on Whatman 1 in a descending manner.

| Sample | Paper electrophoresis pH 3.5 (mm) | pH 6.5 (mm) | Paper chromatography Butanol-acetic acid-water (4:1:5) $R_f$ |
|---|---|---|---|
| Oxytocin | −93 | −105 | .61 |
| Human oxytocic component | −93* | −105 | .62 |

* Tested for activity from unsprayed strip and found to have avian depressor activity.

The isolated fractions were characterized by various means. The peak containing vasopressor activity was assayed in both rat and bird. The ratio of activities was about 8 to 1, respectively, which is approximately the same as a sample of arginine vasopressin tested under identical conditions.

Technics of paper chromatography and paper electrophoresis were used to compare isolated samples with authentic samples of oxytocin, lysine and arginine vasopressin. In addition to detection of the components on paper with ninhydrin and bromphenol blue, the

TABLE III. Comparison of Human Pressor Component with Arginine and Lysine Vasopressin. Paper electrophoresis on Whatman 3MM, 22 in long × 4 in wide. Mobility in mm indicates the direction of movement. Paper chromatography on Whatman 1 paper in a descending manner.

| Sample | Paper electrophoresis pH 3.5 (mm) | pH 5.6 (mm) | pH 6.5 (mm) | Paper chromatography (1) | (2) |
|---|---|---|---|---|---|
| Lysine vasopressin | −95 | −137 | −118 | .08 | .26 |
| Arginine " | −93 | −125† | −109† | .104† | .284† |
| Human pressor component | −92* | −128† | −108† | .102† | .295† |

(1) Butanol/acetic acid/water—4:1:5. (2) Butanol/acetic acid/water/pyridine—30:6:24:20.

* Tested for activity on unsprayed strip and found to possess pressor activity.
† Positive test for arginine when sprayed with Sakaguchi reagent(9).

samples containing the vasopressin were also sprayed with the Sakaguchi reagent.

The results indicated that the sample of human oxytocin behaved in a manner identical to that of beef oxytocin after electrophoresis at pH 6.5 (Table II). In the case of the human vasopressin, the behavior on paper chromatography and paper electrophoresis was identical to that of arginine vasopressin (Table III). In addition the spots due to the vasopressin of the human gave a positive color with the Sakaguchi reagent.

Aliquots of each preparation were hydrolyzed in 6 *N* hydrochloric acid and the amino acids were identified by 2 dimensional paper chromatography, with butanol-acetic acid-water (4:1:5) for the first dimension and phenol-water (75:25) for the second dimension. All the amino acids found in oxytocin were present as well as traces of several others. The pattern obtained from the vasopressin hydrolysate showed the presence of only those amino acids found in arginine vasopressin. In addition, the basic residue from the vasopressin was also identified following paper electrophoresis of the hydrolysate (Table IV). At pH 5.6, in pyridine acetate buffer, a spot was obtained from human vasopressin hydrolysate agreeing in mobility with that of arginine and giving a positive test with the Sakaguchi reagent (Fig. 1).

TABLE IV. Paper Electrophoresis of Acid Hydrolysate of Pressor Component. Conditions: pH 5.6, pyridine acetate buffer, 400v, 4-5 ma, 5 hr; Whatman 3MM paper, 22 in long × 4 in wide. Mobility in mm indicates direction of movement.

| Sample | Migration (mm) | Sakaguchi reagent |
|---|---|---|
| Lysine, 10 μg | −130 | Negative |
| Arginine hydrochloride, 10 μg | −121 | Positive |
| Human vasopressin hydrolysate | −19, −120, +88, +103 | " 120 mm |

It will be recalled that a large fraction of the activity remained in the supernatant solution after the proteins were salted-out with sodium chloride. It was necessary to examine this fraction and to determine the identity of the remaining active components. The proteins were removed by precipitation with a final concentration of 10% trichloracetic acid. The supernatant solution was added directly to a column of IRC-50. After removal of trichloracetic acid the components were separated by the gradient elution technic. Although the separated components were far from pure, the behavior of the active material on the column agreed with the results obtained with oxytocin and arginine vasopressin. Additional tests on the isolated fractions substantiated this point.

It is evident that the active components in an extract of posterior pituitary gland of the human can be accounted for by the presence of the known structures for oxytocin and arginine vasopressin. The hormones of human posterior pituitary agree in composition with hormones of beef rather than the hog. It is rather interesting that in the 3 cases so far examined, the oxytocin of the beef, hog and human are the same.

*Summary.* The oxytocic and pressor activities of the human posterior pituitary gland were separated chromatographically after preliminary purification. The behavior of purified components on ion exchange chromatography, paper chromatography and pa-

per electrophoresis indicated that they possessed the properties of oxytocin and arginine vasopressin. The amino acid composition of the oxytocin and vasopressin isolated from the human glands was determined. It is evident that the oxytocin and vasopressin found in the human pituitary are identified as oxytocin and arginine vasopressin.

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## Effect of Cortisone and X-radiation on RNA and Glycogen Content of Rat Hepatocytes.* (24155)

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It was previously observed that DNA content of rat liver nuclei decreased both during cortisone administration and following x-radiation. In the former instance, the fall was progressive with continuing administration of the hormone; in the latter. there was a return to normal 5 days after x-radiation. Furthermore, daily cortisone administration to rats previously exposed to radiation caused a fall in DNA/nucleus and prevented the return to normal values at 5 days. A hypothesis was also presented to account partially for these observations. It was suggested that the apparent losses and increases in DNA/nucleus were due to depolymerization and repolymerization of the DNA(1). It appeared pertinent to study the effects of cortisone and/or x-radiation on the RNA and glycogen content of rat liver.

*Materials and methods.* Male rats of Wistar strain,†, weighing between 100-120 g were fed *ad libitum* on Lablox R diet.‡ Four groups of animals were studied: Group 1 received no treatment and served as control; Group 2 was injected with cortisone intramuscularly (25 mg/day) for one to 5 days and sacrificed after appropriate fast: Group 3 was exposed to x-radiation (1300 r) and killed from one to 5 days after x-radiation; Group 4 was cortisone-treated post-x-radiation; cortisone administration started within one hour after x-radiation and was given for one to 5 days. Details regarding cortisone dosage, frequency of administration, dosage and conditions of x-radiation and other precautionary steps were identical with those described previously(1). All animals were fasted 24 hours before removal of livers and were sacrificed between 9:00 and 10:00 a.m. to obviate diurnal variations in metabolism.

*Cellular disruption and chemical analyses.* At intervals after appropriate treatment, fasted animals were sacrificed and a 20% homogenate of liver pulp was prepared in slightly alkaline saline (containing 2 ml of 0.1 N NaOH/L of 0.85% NaCl W/V). Nucleic acids were extracted from a portion of the brei by procedure of Schneider(2) using hot trichloracetic acid (TCA), following removal of acid soluble materials with cold 5%

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† Carworth Farms, New City, Rockland Co., N. Y
‡ Allied Mills, Peoria, Ill.

TABLE I. Effect of X-radiation and/or Cortisone on RNA and Glycogen Content of Rat Liver.*

| Days after initiation of treatment§ | Cortisone (Group 2) | | X-radiation (Group 3) | | X-radiation and cortisone (Group 4) | |
|---|---|---|---|---|---|---|
| | RNA† | Glycogen‡ | RNA | Glycogen | RNA | Glycogen |
| | mg/liver | | | | | |
| 0‖ | 19.8 ± .6 (12) | 23.8 ± .7 (12) | 19.8 ± .6 (12) | 23.8 ± .7 (12) | 19.8 ± .6 (12) | 23.8 ± .7 (12) |
| 1 | 24.4 ± 1.0 (10) | 74.4 ± 3.0 (10) | 24.4 ± .9 (10) | 49.5 ± 2.4 (10) | 28.6 ± 1.3 (10) | 188.1 ± 10.2 (10) |
| 2 | 28.0 ± 1.2 (10) | 181.4 ± 8.2 (10) | 22.9 ± 1.1 (10) | 71.6 ± 3.1 (10) | 33.1 ± 1.1 (10) | 302.9 ± 10.9 (10) |
| 3 | 27.0 ± 1.5 (10) | 357.5 ±14.6 (10) | 20.9 ± .9 (10) | 105.0 ± 4.2 (10) | 29.5 ± 1.7 (10) | 206.4 ± 10.5 (10) |
| 4 | 22.5 ± 1.1 (9) | 386.0 ±16.6 (9) | 16.4 ± 1.2 (10) | 12.9 ± .7 (10) | 21.6 ± 1.5 (11) | 180.1 ± 8.5 (11) |
| 5 | 21.0 ± .8 (9) | 353.0 ±14.6 (9) | 17.0 ± 1.8 (11) | 20.1 ± 1.5 (11) | 17.9 ± 1.4 (10) | 162.2 ± 8.2 (10) |

* Mean and stand. error of mean. Figures in parentheses refer to No. of experiments.
† RNA—Ribose.
‡ Glycogen values expressed as total reducing carbohydrate.
§ Days after x-radiation or days cortisone administered.
‖ Initial control Group I.

TCA. The TCA extract was analyzed for ribose by the orcinol reaction(3). Glycogen was determined on another portion of brei (4). The accompanying Table uses the term RNA to indicate nucleic acid-ribose, since this was the material determined. Glycogen content is expressed as total reducing carbohydrate. In view of alterations in liver weights following cortisone administration or exposure to x-radiation(1), the RNA and glycogen values are expressed as mg/liver.

*Results.* The data are presented in Table I. Total RNA increased and returned to normal in the last 3 groups of animals. While maximum values were observed on second day in Groups 2 and 4, x-radiation (Group 3) stimulated maximum RNA increase at 24 hours. However, in x-radiation, the increase was smallest, and the values returned to normal earliest. X-radiation, in conjunction with cortisone, showed largest accumulation of RNA, but even in this group of animals, RNA values had returned to normal by fifth day.

Data on glycogen accumulation may also be observed in Table I. X-radiation caused liver glycogen to increase for 3 days following exposure and to decrease sharply on days 4 and 5. Following cortisone administration, however, liver glycogen increased for 3 days and leveled off at very high value on days 3 to 5. When animals previously subjected to x-radiation received cortisone, glycogen content rose but not to the levels observed in animals receiving similar amounts of cortisone without radiation. It is apparent from these data that x-radiation modified the usual rate of accumulation of glycogen induced by cortisone. Acute x-radiation has been reported to decrease drastically liver glycogen content(5).

*Discussion.* It appears worth while to compare alterations on RNA and glycogen content obtained in the present study with those of DNA/nucleus obtained in a previous report(1). Such a comparison is warranted, since experimental conditions in both cases are similar and since these agents caused alteration in DNA content of rat hepatocytes. In animals which received cortisone alone, the maximum increase of RNA was obtained 24 hours prior to the time when a significant fall in concentration of DNA was observed, day 3. However, in animals which received x-radiation or both cortisone and x-radiation, maximum accumulation of RNA was found 48 hours earlier than when the fall in DNA became significant, days 3 and 5, respectively. The rapid increase in RNA content of rat liver to different levels under various conditions of treatment in our investigation would

imply either an increased rate of synthesis or decreased rate of destruction. The increases in RNA content observed might also be nonspecific, since administration of massive doses of estradiol and progesterone(6) to rats and thyroxine to rats(7) or mice(8) produces a similar rise in RNA content of liver. However, failure of RNA concentration to increase beyond the third day after x-radiation and/or cortisone administration can be correlated with onset of a fall in DNA content. This may also be a reflection of interference with intracellular biochemical organization resulting from depolymerization of DNA. Allfrey and co-workers(9) reported that *in vitro* protein synthesis by isolated thymus nuclei, a reaction dependent on structural integrity of DNA, is inhibited when cortisone is added to the reaction mixture.

Accumulation of glycogen in cortisone-treated group reached a peak level (day 4) 24 hours after the fall in DNA had become significant. In animals exposed to x-radiation, the maximum deposition of glycogen in liver occurred on day 3, when DNA/nucleus was also minimum. When maximum accumulation of glycogen was observed in animals receiving both cortisone and x-radiation (day 2), DNA/nucleus was not different from that found in untreated control. In view of lack of certainty concerning the locus of action of cortisone(10-15), as well as paucity of information relevant to effects of x-radiation on enzymes involved in gluconeogenesis, any explanation of the manner by which x-radiation modifies glycogen accumulation by cortisone does not appear pertinent at the present time.

*Summary.* Rats were treated with cortisone, x-radiation and both agents in combination, and the effects noted on RNA and glycogen content of liver. The following observations were made on concentrations of RNA and glycogen relative to that of DNA: (1) Under various conditions of treatment, RNA content increased promptly to different degrees and returned to normal values at different times. Comparison of RNA content/liver in the present study, with those of DNA/nucleus in previous study, indicates that, in animals which received cortisone alone, the maximum increase in RNA was obtained 24 hours prior to a fall in concentration of DNA. However, accumulation of RNA in rats which received x-radiation or both cortisone and x-radiation was 48 hours earlier than when the fall of DNA became significant. (2) X-radiation or cortisone administration caused glycogen accumulation in liver, the former transiently, the latter in more dramatic and prolonged manner. X-radiation, prior to administration of cortisone, modified the response and decreased glycogen accumulation. Changes in glycogen content in contradistinction to RNA could not be correlated with those of DNA.

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# Chronic Catheterization of Carotid and Vertebral Arteries in Cats.* (24156)

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In investigating drug action on the central nervous system, it is sometimes advantageous to introduce the drug directly into the cerebral circulation, by-passing the systemic vessels. Since certain drug effects can be examined only in the unanesthetized state, a technic permitting injection into intact, freely moving animals is essential. In this way, valuable observations can be made upon the neurologic status of the animal, its general behavior, or even its performance in a psychological test situation, and electrical activity of the brain recorded. Furthermore, because drug effects vary with dose, it is helpful to have a permanent preparation in which different doses of drug may be tested repeatedly over weeks with a single animal serving as its own control. In the following a technic is described for permanently implanting carotid and vertebral catheters in cats, with a consideration of how such a preparation can be of value in studying central nervous system drug effects.

*Method.* Carotid catheterization: in cats under pentobarbital anaesthesia, one common carotid artery is exposed aseptically and the largest muscular branch at the level of the thyroid cartilage divided as far distally as possible. The carotid is occluded above and below this branch, which is then slit transversely, and a catheter‡ introduced into the muscular branch and advanced up the common carotid for approximately 1-2 cm. It is then tied firmly in place at the bulb with several ligatures around the muscular branch and the other end brought out, by means of a hubless trocar, through a stab wound between the scapulae. *Vertebral Artery*: a longitudinal incision is made to the left of the midline over the lower neck, and the left subclavian artery and brachial plexus exposed and identified, as well as the thyrocervical trunk, costocervical trunk and the vertebral artery. The axillary artery, costocervical trunk, and thyrocervical trunk are then ligated, the latter as far distally as possible. With the subclavian artery temporarily occluded proximally, the thyrocervical trunk is opened transversely and the catheter inserted into it towards the heart until it enters the subclavian and its end rests about at the point of origin of the vertebral artery, where it is tied in place. Injections are thus made into the subclavian at point of origin of the vertebral, with the other branches of the subclavian in the vicinity (axillary, thyrocervical trunk, and costocervical trunk) tied off. It will be apparent that the left side is suitable for such a procedure anatomically, but the right side is much less so because of the immediate proximity to the vertebral of the right common carotid artery. The external end of the catheter is then attached to a modified polyethylene tubing adapter (Clay-Adams). Its hub is shortened and screw threads made in its base externally. The cavity of the hub is then filled with a soft rubber plug suitable for needle puncture and a specially made collar with a central hole is screwed down over the hub to keep the rubber firmly in place. The apparatus is then mounted in a plastic yoke containing holes through which it may be sewed to the underside of a closely fitting plastic animal jacket. Injections are made by inserting a #27 short hypodermic needle through the diaphragm. It is possible to give the injection (usually 0.1 cc-0.2 cc) quickly without disturbance, and to withdraw the needle while observing and testing the animal; or one may attach the catheter to a long polyethylene hose of the same caliber, using a short section of #27 needle as a link, and

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† U.S.P.H.S. Senior Research Fellow.

‡ Size PE 10 Clay-Adams polyethylene tubing prepared with a small bulb 2 cm from one end and coated with Siliclad (Clay-Adams) to minimize clotting. Catheters were sterilized in benzalconium chloride and washed thoroughly with sterile saline.

make intracarotid injections from a distance in the undisturbed cat. The catheter is flushed with sterile saline before and after injections of the drug and is filled with heparin at the end of each test period. Electrodes have been implanted over the cerebral cortex of the same animal, making it possible to record the EEG during the drug action.

*Results.* A number of drugs have been employed in this preparation, and the neurological, behavioral, and EEG effects noted. The effects of intracarotid sodium pentobarbital will serve as an instructive example.

Intracarotid sodium pentobarbital was given over a wide range of doses to establish threshold, minimal effects, etc. Because of its relatively slow rate of elimination, only one injection was given each day. In cats weighing 3-4 kg, doses of 0.2 mg or less gave no effect. Minimal effective doses (0.25-2.0 mg) produced a characteristic syndrome of circling to the side of the injection, accompanied by neglect of the contralateral homonymous visual field. Certain of the cats invariably showed simultaneous relaxation of the ipsilateral nictitating membrane. Duration of symptoms depended on dose. Doses in the intermediate range (2.0 to 20.0 mg) produced the above effects plus weakness of contralateral extremities, manifested by feeble withdrawal from noxious stimuli and inadequate hopping and placing reactions. Larger doses (20-30 mg) produced evidence of marked motor impairment of the contralateral side—a rather complete hemiplegia with increased extensor tone in the contralateral fore and hind limbs, and increased deep tendon reflexes. The cat fell to the contralateral side due to lack of support. This was accompanied by pronounced curvature of the body to the ipsilateral side and adversion of the head to that side as well.

With doses in the intermediate range, generalized effects became apparent in a minute or two, once the drug had sufficient time to recirculate and affect the rest of the brain. This usually manifested itself by pronounced playfulness and staggering gait, sometimes accompanied by drowsiness but never by excitement. With larger doses the cat became


FIG. 1. The 2 channels record EEG from homologous areas of each hemisphere and show marked EEG asymmetry resulting from administration (in 1 mg doses) of 9 mg of pentobarbital into left common carotid artery. Record from left hemisphere shows a series of spindle bursts, as seen in moderately deep barbiturate anaesthesia. Record from right hemisphere is that of alert wakefulness and has not changed significantly from control period taken before drug administration.

stuporous or unconscious. After the largest doses employed (75 mg) unconsciousness supervened so rapidly that there was scarcely time to make any useful observations.

It was possible to record the EEG while the injections were being made remotely, with the cat undisturbed but observed. Sodium pentobarbital produced marked EEG asymmetry, with the side ipsilateral to the injections first showing barbiturate induction activity (high voltage fast waves, 18-20/sec.), then barbiturate sleep (slow waves and spindles). During this time, the record from the contralateral hemisphere was that of the normal waking cat (Fig. 1). When injections were stopped, the process reversed itself, passing back from "sleep" through "induction" to normal. Recovery was much more rapid than that observed after intravenous injection. EEG effects correlated well with behavioral observations, since the animal showed contralateral hemiparesis when EEG effects were maximal; both recovered together.

*Complications of the method.* If blood passes back up the catheter and remains for any length of time, it may clot and permanently occlude the catheter. Hence it is important to prevent leaks in the system and to insure that after each experimental session the catheter is filled with heparin solution. Although the cats are not particularly inclined to disturb the catheter where it is exposed externally, they may scratch the region or attempt to groom it, so that it is essential to avoid any loops in which the animal may catch its paw. Consequently, the external end of the catheter may be plugged with wire

and left free, or attached to the special adapter and protected beneath a plastic coat. We have had no infections and there has been no suggestion of embolization of the brain at time of injection or evidence of such upon inspection of the brain at autopsy.

In those animals in which intra-arterial clotting ultimately took place, the thrombus has been located within the carotid a little beyond the tip of the catheter, rather than at the point of entrance of the catheter into the carotid where it might have been anticipated. In one instance, the unilateral drug effects disappeared and were replaced by evidence of discomfort on injection. The animal was sacrificed and india ink injected into the catheter immediately before death. A thrombus occluded the common carotid just beyond the catheter tip; the ink had traveled centripetally to the junction of the 2 carotids, up the opposite carotid and into both hemispheres of the brain by crossing over in the circle of Willis. In a second instance, the threshold for the unilateral effects of several different drugs suddenly increased 4-5 fold, while there was no change in the cat's susceptibility to their general (recirculation) effects. At autopsy, it was found that the internal carotid was thrombosed at its origin, and the channel to the external carotid (and thus through the internal maxillary artery and carotid plexus to the circle of Willis through the orbital fissure) was partly occluded as well. Apparently full patency of both internal and external carotid arteries is important in obtaining low-threshold unilateral effects, but that if the occlusion is only partial, unilateral effects can still be obtained but with corresponding decrease in unilateral versus generalized drug effects.

Certain side effects of the drug injection, such as relaxation of the ipsilateral nictitating membrane and licking and gagging, appear to be due to irrigation of the superior cervical ganglion and the tongue and throat respectively. By placing the tip of the catheter beyond the source of blood supply to these structures, one can observe central neurological effects alone.

It was feared that in the case of the indirect vertebral catheterization, ligation of the left subclavian artery and costocervical and thyrocervical trunks might produce ischaemia of the left forelimb. This has not been the case, but a reversible paraparesis involving both lower extremities has been noted, apparently from diminution of the blood supply to the spinal cord at the cervicothoracic junction.

*Discussion.* Use of the carotid route for administration of neurotropic drugs to unanaesthetized man was begun by Wada(1). Injections were made by percutaneous carotid puncture and resulting neurological and psychological alterations observed. This method has promise but is naturally limited to safe drugs selected primarily for possible therapeutic effect. Recently, this technic has shown itself especially useful in the lateralization of speech function in man(2).

The intra-arterial route has been employed in experimental animals for a variety of purposes. The carotid loop technic permits intracarotid injections in trained, unanaesthetized dogs(3,4,5). A single injection of the long-lasting anticholinesterase, DFP, into the rabbit carotid produced significant effects upon the EEG(6) and behavior(7). These injections were made under local or short-acting barbiturate anaesthesia, and the drug effects lasted long enough to allow examination of the freely moving or curarized animal. For observation in the acute preparation of short-lasting drug effects or for observing the effects of several repeated injections, a carotid T-tube or catheter has been employed(8,9,10). Subsequent use has been made of single or repeated intracarotid or intravertebral injections in animals immobilized by brain stem section or curarization(11,12,13,14), or anaesthesia(14,15,16).

Short-term chronic carotid catheterization in the rat was employed in the study of ADH release(17), and a technic for long-term chronic carotid catheterization has been successfully devised by Rudolph and Paul(18) for the repeated measurement of intra-arterial pressure in dogs. An indirect technic for intravertebral injection in guinea pigs was employed by Diamant(19), and involved retrograde (centripetal) injection into either caro-

tid, with both carotids occluded further distally. This was done under local anaesthesia and sometimes a single animal was used more than once, but the method carried the disadvantage that it required injection of a large fluid volume with simultaneous clamping of both common carotids, measures which alter cerebral hemodynamics profoundly.

With our method, we have found it possible to prepare cats with chronic catheters and to maintain them for long periods. Such preparations afford certain distinct advantages in the study of drug action upon the nervous system. The preparation is very sensitive. Whereas a drug-induced decrease of total motor output of both cerebral hemispheres of perhaps 10% would scarcely be detectable merely by observing the animal, such a decrease in only one hemisphere becomes readily apparent, since by imbalance of the output, the animal turns to one side when it attempts to walk.

The presence of a blood brain barrier to different agents may be studied. The total dose of drugs given systemically to the intact animal may be limited by undesirable side effects (such as respiratory paralysis in the case of curare) or by very rapid destruction or inactivation. The present method makes it possible to administer large doses to the brain with comparatively little systemic effect, and the relative capacity to penetrate the blood brain barrier is increased. Furthermore, since the drug may also irrigate the superior cervical ganglion, which lacks this barrier, and thus affect the nictitating membrane, it is possible to compare the effects of the drug upon central and peripheral synapses simultaneously.

The latency within which drugs act upon the brain may be measured precisely, since the drug blood level in the brain is very high initially during a single circulation time, and falls sharply immediately thereafter. Furthermore, the recovery rate of nervous tissue may be ascertained, since after the injection the concentration gradient is away from the brain only.

The locus of drug action may be more precisely determined, since it is possible to administer drugs predominantly to the hemispheres, with little reaching the lower brain stem, or vice versa. Furthermore, since the hemispheral action is predominantly unilateral, the other hemisphere serves as a control against systemic or non-specific effects. Some drugs may act upon the nervous system only indirectly, by releasing other chemical substances or after being changed chemically themselves. Since these all involve recirculation, this phenomenon will be revealed by the absence of lateralized effects.

Since a single animal can be examined repeatedly over a period of months, it is possible to give different doses of a drug and establish dose-response curves, or to administer different drugs and make quantitative comparisons. Lastly, since the animals are healthy and freely moving it is possible to test them in a variety of ways, neurologically and psychologically, to photograph their movements and behavior, and to record from the surface or depths of the brain by means of implanted electrodes.

*Summary.* 1. A technic is described which makes it possible to administer drugs into the carotid or vertebral circulations of intact, freely moving cats repeatedly over a period of weeks or months. Observations and photographs have been made of the resulting neurological or psychological effects, accompanied in some instances by simultaneous electrical recording from the cortex and deeper structures. 2. The effect of intracarotid sodium pentobarbital is described as an example. This has produced ipsiversive circling, relaxation of the ipsilateral nictitating membrane, and contralateral paralysis, spasticity, and homonymous hemianopsia, the effects varying with dose. Unilateral EEG changes, characteristic of barbiturates, accompany the neurological symptoms and signs. The latency of onset is very short and all the effects are fully reversible in 5-10 minutes. The advantages of the technic are discussed.

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## Immunochemical Studies with Radioactive Isotope, Similarity and Difference Between Serum Vitellin and Lipovitellin. (24157)

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The serum of laying hens was first distinguished from that of non-laying hens and of cocks by Sasaki(1) who used immunological precipitin tests. Roepke and Bushnell(2) using immunological methods detected traces of protein similar to ovovitellin in serum of cocks, and more in laying hens. Lakowski (3) found in plasma of laying hens a phosphoprotein which he named serum vitellin since its chemical nature is similar to ovovitellin. Recently Hosoda and his associates(4), using immunological technic determined the vitellin content in blood serum of hens and found that its titer corresponds to ovarian activity of hens. Wormall and his co-workers (5,6) studied the chemical composition of lipovitellin-antilipovitellin precipitates using radioactive isotopes. The present work was undertaken to elucidate the relationship between serum vitellin and egg yolk lipovitellin, using immunochemical methods with radioisotope.

*Materials and methods.* Antigens. 350 $\mu$c $P^{32}$ as $Na_2HPO_4$ was injected subcutaneously into laying hens. After 24 hours the hens were sacrificed, and $P^{32}$-containing serum and yolk solution taken up as antigens in 10% NaCl. *Antiserum* to serum of laying hens was prepared by immunizing rabbits with non-radioactive serum of laying hen, and absorbing the species-specific antibodies with cock serum. This type of absorbed-antiserum has been used by Hosoda *et al.*(4) for determination of serum vitellin. Anti-yolk serum was prepared by immunizing rabbits with a suspension of non-radioactive yolk. This antiserum had a considerable amount of species-specific antibodies, absorbed with cock serum. Lipovitellin used as antigen was prepared as described by Chargaff(7). Antilipovitellin serum was prepared by immunizing rabbits with a suspension of nonradioactive lipovitellin. Anti-lipovitellin serum also had considerable amount of species-specific antibodies, which were absorbed with cock serum. Phenol was added in a concentration of 0.25% to all antisera for preservation, and these antisera were stored at 3-5°C. *Precipitin tests.*

Each 1 cc of $P^{32}$-containing serum and 5% (W/V) solution of egg yolk in 10% NaCl, used as antigens, were mixed with 20 cc of antiserum, the mixtures were kept 2 hours at 37°C over-night at 0-4°C in refrigerator, then centrifuged 10 min. at 2-4°C at 4000 rpm. For each serological test, complete disappearance of antigen was confirmed by interfacial precipitin reactions on the supernatant solution. In further experiments, NaCl was added to each of $P^{32}$-containing serum and antisera to give the same salt concentration (10%) as in egg yolk solution. In another case, yolk solution in 10% NaCl was mixed with 10%-NaCl-added antisera. The succeeding procedure was the same as described above. The experiments with yolk solution were performed only to avoid the possibility of precipitation of lipovitellin by reduction in salt content; however, no precipitate was observed when 1 cc of a 5% solution of yolk in 10% NaCl was diluted with 20 cc of non-immunized serum of rabbit or cock. *Phosphorus determinations and radioactivity measurements.* All of the precipitates were washed twice with small amounts of ice cold distilled water to remove non-specific substance, and all the supernatants were fractionated by the method of Schneider(8). After the acid soluble fraction was extracted with cold trichloroacetic acid, the lipid fraction was removed by extraction with hot ethanol-ether (7:3 by vol.), and the nucleic acid fraction was extracted with hot trichloroacetic acid. The residue was regarded as the protein fraction. Lipid P and protein P in serum and yolk used as antigen were determined by the method of Fiske and Subbarow(9). Measurement of radioactivity of $P^{32}$ in each fraction was made with a Geiger-Muller tube with a scaler. P contents in lipid fraction and protein fraction of precipitates were calculated by comparing radioactivity of fractions with that of original serum and yolk used as antigen(6,10).

*Results.* $P^{32}$-containing serum and yolk obtained from laying hens 24 hours after injection of $P^{32}$, were fractionated by immunological method described, into the precipitate and the supernatant. Distribution of radioactivity of $P^{32}$ in phosphorus partitions of the precipitates, the supernatants obtained with serum of laying hen and yolk solution in 10% NaCl and various antisera to those substances containing no extra salt, is given in Table I. Antisera to the serum of laying hen absorbed with cock serum, anti-yolk serum, anti-yolk serum absorbed with cock serum, anti-lipovitellin serum, and anti-lipovitellin serum absorbed with cock serum were used. In all cases acid soluble P was not detected in the precipitates, but it was present in supernatant solutions, and in any case nucleic acid P was not detectable in precipitates and supernatants. When serum of laying hens was used as antigen, each of the absorbed antisera described above precipitated one-half of the radioactivity of $P^{32}$ in lipid P fraction, while unabsorbed antisera precipitated two-thirds of it in the same fraction. On the other hand, approximately 95% of radioactivity of $P^{32}$ in protein P fraction was always present in precipitates. The precipitates obtained from mixtures of sera of laying hens and from unabsorbed antisera, contained considerably more phospholipin on the basis of phosphoprotein than did precipitates obtained from mixtures of sera of laying hens, and absorbed antisera.

When yolk solution in 10% NaCl was used as antigen, each of the unabsorbed antisera precipitated three-fourths of the radioactivity of $P^{32}$ in lipid P while precipitates obtained with absorbed antisera contained 55% of the activity. Only 22-36% of the radioactivity of $P^{32}$ in protein P was precipitated in every case, while 95% was precipitated when serum of laying hens was used as antigen. Yolk-unabsorbed antisera precipitates contained considerably more phospholipin on the basis of phosphoprotein than did yolk-absorbed antisera precipitates.

No difference in distribution of radioactivity of $P^{32}$ in each fraction was observed among the 3 kinds of unabsorbed antisera. The same phenomenon was observed among absorbed antisera.

Composition of precipitates obtained from mixtures of serum and yolk of laying hens, and of various antisera to these substances, is

TABLE I. Distribution of Radioactivity of $P^{32}$ in Phosphorus Partitions of Precipitates and Supernatants Obtained with Serum and Yolk* of Laying Hen and the Various Antisera† to These Substances.

| Antigen fractions | Antiserum to laying hen serum | | Absorbed‡ antiserum to laying hen serum | | Anti-yolk serum | | Absorbed‡ anti-yolk serum | | Anti-lipovitellin serum | | Absorbed‡ anti-lipovitellin serum | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ppt.§ | sup.§ | ppt. | sup. | ppt. | sup. | ppt. | sup. | ppt. | sup. | ppt. | sup. |
| *Serum* | | | | | | | | | | | | |
| Acid soluble ph.‖ | 1.9 (19 ) | 7.5 (81 ) | .2 (1.2) | 14.6 (98.8) | 1.1 (6.7) | 15.3 (93.3) | .7 (4.2) | 15.9 (95.8) | .8 (5.5) | 13.8 (94.5) | .4 (2.6) | 15 (97.4) |
| Lipid ph. | 36.2 (60.5) | 23.6 (39.5) | 29.3 (50.8) | 28.4 (49.2) | 33.1 (63.8) | 18.8 (36.2) | 24.8 (45.8) | 29.3 (54.2) | 39.1 (71.3) | 15.7 (28.7) | 29.4 (55.2) | 23.9 (44.8) |
| Nucleic acid ph. | 0 | 0 | .6 | 0 | .8 | .2 | 1.1 | .1 | .9 | 0 | .9 | .3 |
| Protein ph. | 30.0 (97.7) | .8 (2.3) | 25.3 (94 ) | 1.6 (6 ) | 29.6 (96.4) | 1.1 (3.6) | 26.7 (95 ) | 1.4 (5 ) | 28.8 (97.3) | .8 (2.7) | 28.9 (96 ) | 1.2 (4 ) |
| Total | 68.1 | 31.9 | 55.4 | 44.6 | 64.6 | 35.4 | 53.3 | 46.7 | 69.7 | 30.3 | 59.6 | 40.4 |
| *Yolk* | | | | | | | | | | | | |
| Acid soluble ph. | 0 | 5.1 | 0 | 7.7 | 0 | 8.5 | 1.7 | 9.3 | .2 | 7.9 | .7 | 9.6 |
| Lipid | 48.2 (71.1) | 19.6 (28.9) | 38.7 (56.8) | 29.4 (43.2) | 50.2 (75.1) | 16.6 (24.9) | 33.4 (51.4) | 31.6 (48.6) | 52.3 (80.6) | 12.6 (19.4) | 40.1 (63.8) | 22.7 (36.2) |
| Nucleic acid ph. | 0 | 0 | 0 | 0 | .3 | 0 | 0 | .6 | 1 | .8 | .4 | 0 |
| Protein | 7 (26) | 20 (74) | 8.7 (36 ) | 15.5 (64 ) | 5.4 (22.1) | 19 (77.9) | 6.6 (28.1) | 16.9 (71.9) | 6.2 (24.7) | 18.9 (75.3) | 8.2 (30.9) | 18.3 (69.1) |
| Total | 55.2 | 44.7 | 47.4 | 52.6 | 55.9 | 44.1 | 41.7 | 58.4 | 59.8 | 40.2 | 49.4 | 50.6 |

* Solution in 10% NaCl. † Not containing any extra salt. ‡ Absorbed with cock serum.
§ ppt. = precipitate; sup. = supernatant. ‖ ph = phosphorus.
Figures in parentheses refer to percentage composition in each fraction.

shown in Table II. The ratio lipid P : protein P in the precipitates obtained with sera of laying hens and the absorbed antisera was 1.1:1, and 1.4:1 in those obtained with sera of laying hens and unabsorbed antisera. This ratio was 4.5:1 in the yolk-absorbed antisera precipitates, and 8:1 in yolk-unabsorbed antisera precipitates.

When NaCl was added to sera of laying hens and to antisera to a concentration of 10%, lipid P content of precipitates diminished to 60% of precipitates obtained with sera and antisera which did not contain extra salt. Protein P content of precipitates obtained from mixtures of yolk solution in 10% NaCl and the 10% NaCl-added antisera, showed a similar tendency. A reduction of lipid P content to 70-80% of that of precipi-

TABLE II. Composition of Precipitates Obtained with Serum and Yolk of Laying Hens and Various Antisera to These Substances.

| | Antigen | | Antisera | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Antiserum to laying hen serum | | Absorbed* antiserum to laying hen serum | | Anti-yolk serum | | Absorbed* anti-yolk serum | | Anti-lipovitellin serum | | Absorbed* anti-lipovitellin serum | |
| | LP | PP | LP | PP | LP | PP | LP | PP | LP | PP | LP | PP | LP | PP |
| Serum | 68 (1.6) | 88 (1) | 96.5 (1.2) | 81.7 (1) | 84.0 (1.1) | 75.1 (1) | 106.5 (1.3) | 80.0 (1) | 82.0 (1.1) | 74.0 (1) | 127.5 (1.6) | 79.0 (1) | 92.8 (1.2) | 76.5 (1) |
| Yolk | 172 (2.5) | 68 (1) | 122.3 (6.9) | 17.7 (1) | 102.0 (4.4) | 23.3 (1) | 117.2 (8.4) | 14.0 (1) | 80.5 (4.8) | 16.9 (1) | 126.0 (8.0) | 15.8 (1) | 86.3 (4.6) | 18.8 (1) |

* Absorbed with cock serum. LP = Lipid phosphorus; PP = Protein phosphorus.
Figures in parentheses showed ratio lipid P : protein P in precipitates All weights are terms of $\mu$g per cc of antigen used (serum: 1 cc, yolk: 1 cc of 5% (W/V) egg yolk solution).

tates obtained with yolk solution in 10% NaCl and antisera containing no extra salt was observed.

*Discussion.* The data showed that each antiserum to serum of laying hens (absorbed with cock serum and unabsorbed), to yolk (absorbed and unabsorbed), and to lipovitellin (absorbed and unabsorbed) precipitated 95% or more of protein P in serum of laying hens, but only about 25% of that in the yolk dissolved in 10% NaCl. It is noted that phosvitin is dissociated from yolk lipovitellin in 10% NaCl solution, and is recombined with lipovitellin when it is reprecipitated(11). Mecham and Olcott(12) and Francis(11) stated that 75% of protein P of vitellin appeared to be due to phosvitin. Furthermore this substance has been shown not to react with antisera to lipovitellin or to vitellin(11). It may be due to the presence of phosvitin dissociated from lipovitellin in 10% NaCl solution, that only 25% of protein P in yolk solution in 10% NaCl can be precipitated with the antisera. However, it seems less probable that in serum of laying hen phosvitin combines with serum vitellin because the present experiments demonstrated that, when salt was added in 10% concentration to serum of laying hens, the antisera still precipitated 95% of protein P in the serum. In other words, no dissociation of phosphoprotein from serum vitellin was observed. Possibly there exists some difference between the structure of serum vitellin and that of lipovitellin in egg yolk, and the serum vitellin may be chemically different after passage through the granulosa of the follicle.

In this study, it was observed that unabsorbed antisera precipitated considerably more phospholipin on the basis of phosphoprotein than did antisera absorbed with cock serum, regardless of whether the serum of laying hens or egg yolk was used as antigen. These results led to the interpretation that unabsorbed antisera precipitate phospholipo-P-free or nearly free protein present in serum of laying hens, which has a similar antigenicity to the substance present in cock serum.

The present study showed that the mean ratio of lipid P to protein P was 1.1:1 and 1.4:1, in precipitates obtained with sera of laying hens and absorbed antisera and those obtained with sera of laying hens and the unabsorbed antisera, respectively. This ratio becomes 4.5:1 in yolk-absorbed antisera precipitates, and 8:1 in yolk-antiyolk or antilipovitellin precipitates. Banks *et al.*(6) reported that the ratio lipid P to protein P in the lipovitellin-antilipovitellin precipitates was 5:1, compared with 1.6:1 for the sample of lipovitellin used. The difference between the precipitates and lipovitellin used as antigen observed by them may be also ascribed to the dissociation of phosvitin having no antigenicity.

The present study demonstrated that injection of serum of laying hens, egg yolk, and lipovitellin into the rabbit produced an antibody which precipitated the same kind of lipoprotein as far as phosphorus is concerned. After the species-specific antibody was excluded, the remainder of the antibody in each case, precipitated whole phosphoprotein combining phospholipin in serum of laying hens; however, in a solution of yolk in 10% NaCl, it appeared to precipitate only non-phosvitin phosphoprotein combining phospholipin. Thus, it is concluded that the structure of phosphoprotein (serum vitellin) combining phospholipin, present in serum of the laying hen, is similar to that of lipovitellin in egg yolk, though there is some difference between them.

Francis and Wormall(5) reported that both lipovitellin and lipid-free vitellin injections produced antisera capable of precipitating lipovitellin. Furthermore the specific antigenic substance in this reaction is greatly affected at 70°C, and completely destroyed at 80°C(13). These results strongly suggest that the antigenicity of lipovitellin may be due to the protein component of the substance. However, phosvitin contained in lipovitellin and accounting for 75% of protein P of vitellin, has been shown not to react in precipitin reactions with anti-lipovitellin serum or antivitellin serum. Injection of phosvitin into rabbits does not produce sera capable of reacting in precipitin reactions with phosvitin, or lipovitellin(11). The antigenicity of lipo-

vitellin would therefore appear to be due to the non-phosvitin component of the protein.

*Summary.* With the help of radioisotope, immunochemical studies on the similarity and differences between serum vitellin and yolk lipovitellin, were made. When $P^{32}$-containing serum of laying hens was used as antigen, each antiserum to the serum of laying hens (absorbed with cock serum and unabsorbed, to yolk, absorbed and unabsorbed, and to lipovitellin, absorbed and unabsorbed), precipitated 95% of the radioactivity of $P^{32}$ in protein P fraction, and 50-65% in lipid P fraction. On the other hand, when $P^{32}$-containing yolk solution in 10% NaCl was used as antigen, these antisera precipitated only 22-36% of radioactivity in the former fraction, and 55-80% in the latter fraction. The ratio of lipid P to protein P in the precipitates obtained with the sera of laying hens and the above described antisera was 1.1-1.6:1, and, in those obtained with yolk and the antisera was 4.5-8:1, compared with 1.6:1 for the lipovitellin used. Injection of the serum of laying hens, yolk and lipovitellin into rabbits produced antibodies which precipitated a similar lipoprotein.

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## Preferential Reduction of Conjugated Bilirubin to Urobilinogen by Normal Fecal Flora.* (24158)

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In earlier experiments in which crystalline bilirubin was added to broth cultures of normal fecal bacteria, it was evident that the proportion reduced to urobilinogen was quite small(1). The possibility existed that these poor conversions depended upon use of free, non-polar bilirubin rather than polar bilirubin glucuronide, the conjugated form of bilirubin present in the bile(2). To examine this possibility the following experiments have been carried out.

*Methods.* A crude concentrate of bilirubin glucuronide was prepared as follows: Fresh human fistula bile was collected in a container having sufficient saturated ammonium sulfate solution to provide at least 50% saturation at end of collection. The vessel was agitated from time to time during collection to allow mixture and precipitation of the pigment. Bile and ammonium sulfate mixture was then filtered through a large amount of Hyflo (diatomaceous silica)† on a Buchner funnel, the Hyflo first having been packed on coarse filter paper with water. Hyflo was also mixed with bile and ammonium sulfate solution before it was poured onto the funnel and the packed Hyflo on funnel was moistened with saturated ammonium sulfate solution before mixture was applied. The packed material

* Aided by contract with Surgeon General's Office, U. S. Army.

† Johns-Manville Hyflo-Supercel.

was repeatedly washed with 50% ammonium sulfate solution. With some samples of bile, a gummy mass was noted at this stage, which was much more difficult to handle. The material on the Buchner funnel was packed fairly dry and extracted repeatedly with small volumes of acetone to remove most of the water. The entire mass was then transferred to a large mortar where it was ground successively, first with small amounts of acetone, next ethyl ether and finally chloroform. The latter removes considerable bilirubin, presumably a fraction which has become deconjugated during above procedure. The final dry powder consists mainly of Hyflo with adsorbed bilirubin glucuronide. Traces of bile acids and other impurities, including biliverdin, are also present. Most of the bilirubin adsorbed on the Hyflo is of the prompt direct type, reacting with the diazo reagent within one minute. The bilirubin glucuronide is readily removed from Hyflo simply by elution with water. After hydrolysis of its azopyrryl compound in 10% HCl for one hour at 100°, and paper chromatography, glucuronic acid was readily demonstrated by the method of Dische (3). The Hyflo powder with adsorbed bilirubin glucuronide keeps well at 4°, bilirubin concentration remaining essentially unchanged over long periods.

In a number of experiments the above described bilirubin glucuronide was compared with crystalline bilirubin in respect to ability of normal fecal flora to reduce it to urobilinogen in broth cultures. Varying amounts of broth, fecal inoculum and bilirubin were used (Table I). The bilirubin was either crystalline, or crude glucuronide as above described or as present in fresh bile. The media consisted of 10 g yeast extract/liter. The fecal inoculum was first incubated with broth for varying periods, usually 12 hours, in sidearm Erlenmeyer flask evacuated by ordinary water pump. As seen in Table I, Exp. 5 A, B and C, there is some evidence that preliminary incubation for 6-12 hours before adding bilirubin, promoted its reduction. pH was adjusted to 7.5 to 8.0 by addition of 10% NaOH, after which the bilirubin solution was added, the flask reevacuated and incubated for 24 hours. pH was readjusted with NaOH about midway in this period, then adjusted to 4-5 (acetic) after which the entire broth culture was extracted 3 times with petroleum ether (B.P. 30-60). The filtered, pooled petroleum ether was subjected to $I_2H_2O$ method previously described(4) for conversion of urobilinogen to urobilin and extraction.‡ Total urobilin group was transferred, as the hydrochloride, to $CHCl_3$. The naturally occurring urobilin group includes varying proportions of levorotatory (l-) Stercobilin, inactive (i-) Urobilin, and dextrorotatory (d-) Dehydro-Urobilin. The problem of terminology will be considered in more detail in a separate communication. The abbreviations l-S, i-U and d-DU are employed to designate the 3 non-isomeric compounds above. Total urobilin was determined in Evelyn colorimeter with a 490 filter against standard plot of optical density of (l-) stercobilin and (i-) urobilin, which have essentially similar absorption. A sufficient volume to provide 200 mg. was then concentrated to dryness on water bath, the residue dissolved in methyl alcohol and this solution subjected to ferric chloride oxidation as described elsewhere. It suffices to note here that the reaction mixture is adjusted to pH 4.0 with sodium acetate solution, and extracted repeatedly with ethyl ether. The latter is washed repeatedly with water, the water washes being returned to the aqueous phase. The combined aqueous solution contains much of whatever l-S was initially present, as this is relatively stable while products of oxidation of the i- and d- compounds remain in the ether. They are readily extracted with 1.5 N HCl the spectral distribution curve of which is then recorded with Beckman DK spectrophotometer.

In the $FeCl_3$ reaction i-U yields a mixture of mesobiliviolin-mesobilirhodin (abs. max. 560 m$\mu$) with smaller amounts of glaukobilin (abs. max. 650 m$\mu$); d-DU yields mainly glaukobilin; l-S neither, its original absorption at 492 m$\mu$ easily recognizable. The approximate composition of initial urobilin

‡ With certain samples of feces, only indol and no urobilinogen was observed.

TABLE I. Urobilinogen Formation from Three Bilirubin as Contrasted with Bilirubin Glucuronide, in Broth Cultures of Fecal Flora.

| Exp. No. | Feces inoculum used | | | | ml broth | Amt (mg) & type bilirubin or urobilin added | Hours of incubation | | Total urobilin group, mg | Approx. % | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | g | No. | Approx. % l-S | i-U | | | Before adding bilirubin | After bilirubin | | d-DU | i-U | l-S |
| 1 A | 1. | I | 70 | 30 | 800 | 0 | 12 | 24 | .09 | | | |
| B | " | | " | " | " | 62 B. G. | " | " | 21.06 | 80 | 20 | |
| C | " | | " | " | " | " cryst. B. | " | " | 14.10 | 75 | 25 | |
| 2 A | .5 | II | " | " | 200 | 0 | " | " | .04 | | | |
| B | " | | " | " | " | 20 B. G. | " | " | 8.83 | 100 | | |
| C | " | | " | " | " | " cryst. B. | " | " | .5 | 20 | 40 | 40 |
| D | " | | " | " | " | " (fresh bile) | " | " | 10.6 | 40 | 60 | |
| 3 A | " | III | 85 | 15 | " | 10 B. G. | " | " | 3.5 | | 10 | 90 |
| B | " | | " | " | " | " cryst. B. | " | " | 4.1 | | | 100 |
| 4 A | .25 | IV | 70 | 30 | " | " B. G. | 8 | " | .46 | 100 | | |
| B | " | | " | " | " | " cryst. B. | " | " | 2.7 | " | | |
| C | " | | " | " | " | " B. G. | " | " | .17 | 60 | 40 | |
| 5 A | .5 | V | 65 | 35 | " | 20 " | 0 | " | 1.9 | 75 | 25 | |
| | | | | | | | | 48 | 1.84 | " | " | |
| B | " | | | " | " | 10 " | 6 | 24 | 3.1 | not determined | | |
| C | " | | | " | " | *Idem* | 12 | " | 4.7 | | 80 | 20 |
| D | " | | | " | " | " + 200 bile salts | " | " | 2.3 | | 100 | |
| E | " | | " | " | " | 10 cryst. B. + 200 bile salts | " | " | .2 | 60 | 30 | 10 |
| 6 A | 1. | V | " | " | 800 | 50 B. G. | " | " | 16.5 | 100 | | |
| B | " | | " | " | " | " cryst. B. | " | " | 5.7 | " | | |
| C | " | | " | " | " | " (fresh bile) | " | " | 19.0 | " | | |
| 7 A | .5 | VI | | | 200 | 10 B. G. | 17 | " | 3.0 | | 40 | 60 |
| B | " | | | | " | " cryst. B. | " | " | 1.7 | | 40 | 60 |
| 8 A | " | VII | | | " | " B. G. | 19 | " | 3.3 | | | 100 |
| B | " | | | | " | " cryst. B. | " | " | 1.9 | | | 100 |
| 9 A | " | VII | | | " | " " i-U | 17 | " | 1.6 | | 10 | 90 |
| B | " | | | | " | " " d-DU | " | " | 3.4 | | 15 | 85 |
| 10 A | .25 | VIII | 100 | 0 | 100 | 5 B. G. | 14 | " | 1.44 | | 50 | 50 |
| B | .5 | | | | " | " " | " | " | 2.38 | | 40 | 60 |
| C | .25 | | | | " | " cryst. B. | " | " | 1.22 | | 20 | 80 |
| D | .5 | | | | " | " " | " | " | .98 | | 40 | 60 |
| E | " | | | | " | 0 | " | " | .097 | | 15 | 85 |
| 11 A | .25 | IX | 100 | 0 | " | 5 B. G. | 15 | " | 1.73 | | 30 | 70 |
| B | .5 | | | | " | " " | " | " | 1.96 | | 40 | 60 |
| C | .25 | | | | " | " cryst. B. | " | " | 1.08 | | 30 | 70 |
| D | .5 | | | | " | " " | " | " | 1.74 | | " | " |
| E | " | | | | " | 0 | " | " | .088 | | | 100 |
| 12 A | .25 | X | 30 | 70 | " | 5 B. G. | 16 | " | 1.37 | | 100 | |
| B | .5 | | | | " | " " | " | " | 1.17 | | " | |
| C | .25 | | | | " | " cryst. B. | " | " | .94 | | 90 | 10 |
| D | .5 | | | | " | " " | " | " | 1.15 | | 100 | |
| E | " | | | | " | 0 | " | " | .148 | | 90 | 10 |
| 13 A | .25 | XI | 80 | 20 | " | 5 B.G. | " | " | 2.04 | 100 | | |
| B | .5 | | | | " | " " | " | " | 1.76 | " | | |
| C | .25 | | | | " | " cryst. B. | " | " | .57 | " | | |
| D | .5 | | | | " | " " | " | " | .46 | " | | |
| E | " | | | | " | 0 | " | " | .02 | | 25 | 75 |

B. G. = bilirubin glucuronide (see text); cryst. B. = crystalline bilirubin (Armour or Eastman); i-U = inactive urobilin; d-DU = d-Dehydrourobilin; l-S = Stercobilin.

group subjected to $FeCl_3$ oxidation is determined by the 2 ratios:

$$\frac{\text{abs. } 492\ (1-S)}{\text{abs. } 560 + 650\ (i-U + d-DU)}; \qquad \frac{\text{abs. } 560\ (i-U > d-DU)}{\text{abs. } 650\ (d-DU > i-U)}$$

This method generally provides reasonably accurate information as to initial proportions and values have usually been in agreement with optical activity measurements.

*Results.* Bacterial formation of urobilinogen group from bilirubin glucuronide was usually more efficient than from crystalline bilirubin (Table I). In 4 experiments (3, 10A, C, 11B, D, 12B, D) the results were regarded as equivocal, although in 3 of these the amounts were slightly larger with conjugated than with free bilirubin. In but one instance (Exp. 4) was the yield clearly greater with free bilirubin. The basis for this difference is not clear. Analysis§ of the comparable experiments in Table I reveals a highly significant difference in favor of the glucuronide ($P<0.03$).

Under above conditions, bacteria from normal feces containing l-S and i-U in varying proportion, at times reduce bilirubin only or mainly to d-DU, again a mixture of d-DU, i-U and l-S is observed. A spectrogram of such a result is seen in Fig. 1. In still other cultures, the i- and l-compounds are formed (Table I). The frequent formation of d-DU by a normal fecal flora supports the view(5,6, 7) that it is a normal product of bacterial reduction of bilirubin in the colon. This is also in accord with the earlier observation(8) that d-DU is readily reduced to i-U, and the present observation (Exp. 9) that normal fecal flora, under above conditions, readily reduce d-DU to i-U and l-S. Starting with bilirubin, bacterial reducing activity may be sufficient to produce only d-DU with small amounts of i-U and l-S (Fig. 1). Starting with d- or i-, however, the reduction is often carried further to the l-form. This is well shown in Exp. 8 and 9 in which it is evident that the fecal flora reduced the added bilirubin to l-S, the added

§ Courtesy of Dr. J. E. Bearman, Div. of Biostatistics, School of Public Health, Univ. of Minnesota.

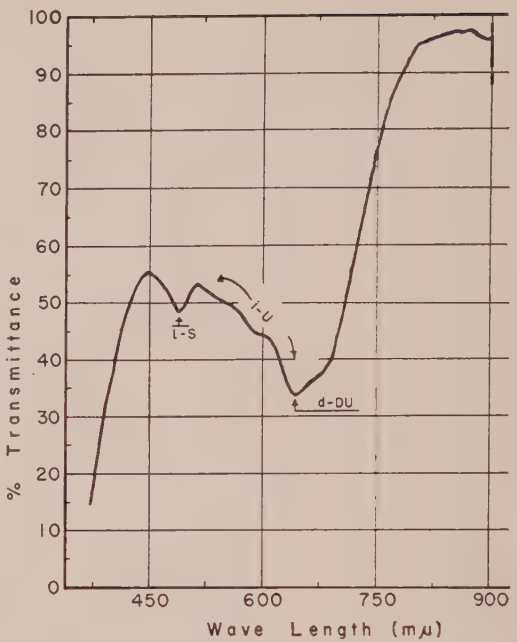


FIG. 1. Spectral distribution of products of $FeCl_3$ oxidation of urobilin group obtained by bacterial reduction of bilirubin glucuronide. Areas of absorption indicated by the arrows relate for l-S to unchanged Stercobilin, for i-U to Mesobiliviolin-Mesobilirhodin, for d-DU to Glaucobilin.

d- or i- forms mainly to l-S. In the one experiment (No. 5) with added bile salts, it appeared that there was hindrance rather than enhancement of conversion to urobilin.

*Discussion.* The basis for greater efficiency of bacterial reduction of the glucuronide as contrasted with free bilirubin has not been determined. It may reside simply in the polar character of the former compound, but the possibility must also be considered that the reduction is incidental to a primary attack on the glucuronic acid.

The present results emphasize bacterial formation of naturally occurring forms of urobilin(ogen) group and close interrelation of d-DU ($H_{40}$); i-U ($H_{42}$) and l-S ($H_{46}$). These designations avoid the objection that use of prefixes d-, i- and l-, as applied elsewhere(5) to the single term urobilin, is unconventional for compounds that are not isomers. The designation d-DU recognizes that this compound is a dehydrourobilin, as shown by our analytical data(6), and emphasized by Gray(7) and Siedel(8). Furthermore, Gray(9) has

recently described a dextrorotatory isomer of i-U, and a racemic DU. While no evidence has yet been presented for their occurrence in nature, their existence enforces the need of distinctive terminology.

Certain additional results given in Table I require brief discussion. In Exp. 2, yield from fresh bile was even greater than from crude glucuronide, yet in Exp. 5 addition of bile salts, either to crystalline bilirubin or crude glucuronide was inhibitory rather than enhancing in its effect. In general, better yields were obtained when there was a 12 hour preliminary growth of bacteria in broth, prior to addition of bilirubin. This is seen most clearly in Exp. 5, Table I.

Recent experiments carried out with S. Gilbertsen, to be described separately, indicate that at least in the normal individual there is a similar basis for failure to note significant increase of fecal urobilinogen after intraduodenal administration of free bilirubin. However, in patients with acholic feces, feeding of above described bilirubin glucuronide has thus far failed to result in formation of appreciable amounts of fecal urobilinogen and other factors must be sought to explain this discrepancy. These studies are being extended.

*Summary and conclusions.* 1. A method of preparing a relatively stable concentrated adsorbate of conjugated bilirubin (glucuronide) from bile, is described. This has been used in a series of experiments in broth cultures of fecal bacteria. 2. A significant and often much greater increase of urobilinogen formation was observed after addition of the conjugated as contrasted with free bilirubin. 3. The urobilinogen group formed in the bacterial cultures consisted of varying proportions of d-Dehydrourobilin, i-Urobilin, and l-Stercobilin. Under the same conditions the fecal flora readily reduced d-DU to i-U, and i-U to l-S.

The technical assistance of Mary Weimer, Irene Bossenmaier and Violet Swenson is gratefully acknowledged.

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## Effect of Route of Injection on Distribution and Excretion of Cortisol-4-C$^{14}$ in the Rat. (24159)

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Distribution and metabolism of cortisol-4-C$^{14}$ in rats has been traced after administration by intravenous, intramuscular, subcutaneous, intragastric, and sublingual routes(1,2, 3). Only minor differences in total amount excreted have been found over periods of 1 to 5 days. A previous report(4) described the fate of cortisol-4-C$^{14}$ in the rat during the first hour after intracardiac injection. In normal animals essentially all of the injected radioactivity was recovered in the GI tract and excreta after 60 min. Extension of these studies in which the hormone was injected intravenously, showed a striking difference from previous results, a difference in distribution as well as a slower rate of excretion. This observation was elaborated since it seemed to have application in the study of the role of

TABLE I. Distribution of Radioactivity, in % of Total Counts Injected, after External Jugular Vein Injection of Cortisol-4-$C^{14}$ into Normal Rats.

| Time after inj. | 5 min. | | | 60 min. | | |
|---|---|---|---|---|---|---|
| Wt, g | 271 | 261 | 263 | 324 | 333 | 327 |
| Dose, $\mu$g | 12.2 | 12.2 | 12.2 | 13.9 | 13.9 | 13.9 |
| , cpm | 48,700 | 48,700 | 48,700 | 55,700 | 55,700 | 55,700 |
| GI tract & excreta | 11.8 | 11.3 | 9.2 | 58.9 | 56.6 | 59.4 |
| Muscle | 43.7 | 43.7 | 38.0 | 23.6 | 12.8 | 21.4 |
| Liver | 20.5 | 23.0 | 24.4 | 10.9 | 13.6 | 11.1 |
| Skin | 16.9 | 13.1 | 17.2 | 9.8 | 11.1 | 8.9 |
| Plasma | 7.1 | 5.5 | 6.9 | 1.0 | 2.2 | 1.1 |
| Kidneys | 3.4 | 3.3 | 4.2 | 1.3 | 1.0 | 1.1 |
| Head | 5.8 | 5.2 | 5.7 | 2.7 | 2.0 | 2.9 |
| All other organs* | | | | 2.8 | 2.9 | 2.9 |
| Total | 109.2 | 105.1 | 105.6 | 110.0 | 102.2 | 107.9 |

* Lungs, spleen, heart, thymus, thyroid, pancreas, adrenals, genital organs, and fat.

adrenal cortical hormones in normal and abnormal physiology. First, radioactivity in the principal organs of intact rats was determined 5 and 60 min. after injection of tracer doses of cortisol-4-$C^{14}$ *via* the external jugular and tail veins, and the results were compared with those obtained after intracardiac injection. Second, the study was repeated in adrenalectomized rats 60 min. after external jugular vein injection of cortisol-4-$C^{14}$ alone and cortisol-4-$C^{14}$ mixed with corticosterone.

*Materials and methods.* The data for intercardiac injection were taken from a previous report(4). For the intravenous injections, 260 to 330 g male Sprague-Dawley rats were used. In the previous studies the rats were fasted overnight prior to intracardiac injection. Since an intact animal fasted overnight was found to have 52.2% of the injected radioactivity in the GI tract and excreta 60 min. after jugular vein injection of 37,900 cpm of cortisol-4-$C^{14}$ (compared to 58.9%, 56.6% and 59.4% in unfasted animals), unfasted animals were used in the studies of intravenous injection. Specific activity of the cortisol-4-$C^{14}$* was 4.08 $\mu$c per mg, 1.0 $\mu$g corresponding to about 4,000 counts per min. (cpm) as determined by our counting technic (4,5). The radiochemical purity had been found to be higher than 94%(6). The adrenalectomies were done 8 days before injection by means of a single mid-abdominal incision without aseptic technic, and the animals were maintained on Purina Lab Chow, saline and water. The right external jugular vein was exposed with a ¾ inch incision. A total of 8.5 to 15 $\mu$g of cortisol-4-$C^{14}$ in 0.7 to 1.0 ml of 10% propylene glycol in saline was injected over 20 to 25 sec. into the tail or external jugular vein under light ether anesthesia using a one ml tuberculin syringe and #27 needle. In the second group of adrenalectomized animals the injected solution contained 194 $\mu$g of corticosterone-$C^{12}$† in addition to the cortisol-4-$C^{14}$. Five or 60 min. after injection the animals were sacrificed by exsanguination from the heart. The muscle, liver, skin, kidneys, and head were handled separately; excreta and bladder were combined with the GI tract; all remaining organs and fat were combined and processed as a group. All tissues were dried and dissolved in formamide; the samples were plated on aluminum planchets with lens paper, counted in a Tracerlab Model SC-50 windowless gas flow counter and the activities corrected as previously described(4,5). The plasma was plated directly. All plasma values refer to a total plasma volume calculated from the weight of the animal(7).

*Results.* In the 5 min. groups (Table I-III) 10% of radioactivity was in the GI tract and excreta. In the intracardiac group the head (after removal of the brain) was processed with the muscles. In the intravenous groups, however, the intact head with the

* The authors are indebted to the Endocrinology Study Section, Dept. of Health, Education and Welfare, N.I.H., Bethesda, Md., for supplying this material through Tracerlab Inc., Boston, Mass.

† The authors are indebted to The Upjohn Co., Kalamazoo, Mich. for supplying this material.

TABLE II. Distribution of Radioactivity, in % of Total Counts Injected, after Tail Vein Injection of Cortisol-4-C$^{14}$ into Normal Rats.

| Time after inj. | 5 min. | | 60 min. | | | | | |
|---|---|---|---|---|---|---|---|---|
| Wt, g | 325 | 299 | 255 | 258 | 273 | 314 | 298 | 323 |
| Dose, μg | 13.0 | 13.0 | 12.4 | 12.4 | 12.9 | 13.0 | 13.0 | 13.0 |
| " , cpm | 51,800 | 51,800 | 49,600 | 49,600 | 51,800 | 51,800 | 51,800 | 51,800 |
| GI tract & excreta | 6.8 | 9.4 | 49.6 | 49.4 | 43.1 | 45.2 | 56.4 | 53.3 |
| Muscle | 42.7 | 36.5 | 18.9 | 17.6 | 18.6 | 22.0 | 18.1 | 13.2 |
| Liver | 20.2 | 18.0 | 6.5 | 6.0 | 6.3 | 10.3 | 5.4 | 7.2 |
| Skin | 16.7 | 13.5 | 12.2 | 14.0 | 13.3 | 9.9 | 9.4 | 9.3 |
| Plasma | 4.0 | 4.3 | 1.5 | 1.3 | 1.6 | 1.4 | 1.3 | 1.7 |
| Kidneys | 3.4 | 4.3 | 1.4 | 1.4 | 1.2 | 1.8 | 1.0 | 1.1 |
| Head | 3.5 | 4.3 | 1.9 | 2.0 | 2.2 | 1.8 | 1.4 | 1.2 |
| All other organs* | 5.0 | 6.0 | | | | 3.0 | 2.0 | 3.0 |
| Total | 102.3 | 96.3 | 92.0 | 91.7 | 86.3 | 95.4 | 95.0 | 90.0 |

* See Table I.

TABLE III. Average Distribution of Radioactivity in % of Total Counts of Cortisol-4-C$^{14}$ Injected into Normal Rats by Various Routes.

| Time after inj. | 5 min. | | | 60 min. | | |
|---|---|---|---|---|---|---|
| Route | Intracardiac* | Ext. jugular vein | Tail vein | Intracardiac* | Ext. jugular vein | Tail vein |
| No. of animals | 5 | 3 | 2 | 3 | 3 | 6 |
| Wt, g | 323 | 265 | 312 | 329 | 327 | 287 |
| Dose, μg | 14.5 | 12.2 | 13.0 | 14.8 | 13.9 | 12.7 |
| " , cpm | 58,000 | 48,700 | 51,800 | 59,300 | 55,700 | 50,700 |
| GI tract & excreta | 11.8 | 10.8 | 8.1 | 98.0 | 58.3 | 49.5 |
| Muscle | 47.2 | 41.8 | 39.6 | 1.9 | 19.3 | 18.1 |
| Liver | 25.3 | 22.6 | 19.1 | 4.3 | 11.9 | 7.0 |
| Skin | 4.4 | 15.7 | 15.1 | | 9.9 | 11.3 |
| Plasma | 6.7 | 6.5 | 4.2 | .7 | 1.4 | 1.5 |
| Kidneys | 3.7 | 3.6 | 3.9 | .8 | 1.1 | 1.2 |
| Head | | 5.6 | 3.9 | | 2.2 | 1.8 |
| All other organs† | 3.7 | | 5.5 | | 2.9 | 2.7 |
| Total | 102.8 | 106.6 | 99.3 | 105.7 | 107.0 | 93.1 |

* Data taken from reference (4). † See Table I.

brain was handled separately. Since the head consisted mainly of attached muscles, it can be seen that total muscle radioactivity content in the 3 groups is about the same. The only striking difference occurred in the skin, (Table III), where radioactivity was higher after both intravenous routes (15.7%, 15.1%) compared to intracardiac injection (4.4%).

Sixty min. after intracardiac injection 98% of the radioactivity‡ had localized in the GI tract and excreta, compared with 58.3% (jugular vein) and 49.5% (tail vein). The portion not excreted 60 min. after intravenous injection was found mainly in the muscle (19 3% and 18.1%) and skin (9.9% and 11.3%).

‡ The comparatively wide range of experimental error(5) should be considered in interpretation of this and all other readioactivity values.

A comparison of our data with that of Ulrich and Long(2) corroborates these findings (Fig. 2). After intracardiac injection we found 6.7%, 4.3% and 0.7% of injected radioactivity in plasma 5, 15 and 60 min. after injection of 14.5 μg of cortisol-4-C$^{14}$(4). Analysis of these results shows a half-time of disappearance from plasma of 17 min. and a residual after 60 min. of 8.6% of the theoretical zero time activity. After injections of 50 μg of cortisol-4-C$^{14}$ into the jugular vein Ulrich and Long found a longer half-time of disappearance from plasma of 26 min. Extrapolation from their half-life curve gives a residual radioactivity of 20% at 60 min. Although in our jugular vein injection studies we have plasma values only for 5 and 60 min., analysis shows a half-time of 25 min. and a residual radioactivity of 19% at 60 min.

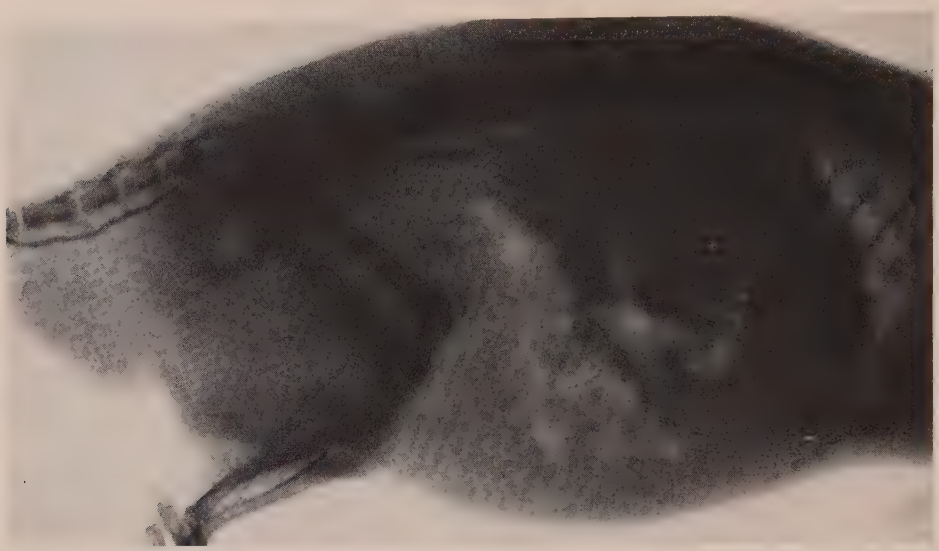

FIG. 1. X-ray during slow (20 to 25 sec.) intrav. inj. of Neo-Iopax.

The high $C^{14}$ content of the skin 5 min. after intravenous injection could have been caused by retrograde flow. X-rays taken while one ml of sodium iodomethanate (50% Neo-Iopax§) was rapidly injected (over 4 to 5 sec) through a #27 needle into the tail vein showed retrograde flow into an abdominal wall vein. However, when the injection was made slowly over 20 to 25 sec (as was done in these studies), all the material went directly into the inferior vena cava (Fig. 1). A similar x-ray study during slow injection of Neo-Iopax into the right external jugular vein showed that the opaque material flowed only into the heart.

Wyngaarden, Peterson and Wolff(1) found 35% and 51% of radioactivity in the bile of 2 cannulated rats one hour after injection of 50 $\mu$g of cortisol-4-$C^{14}$; however, in their studies the steroid was given subcutaneously. Hyde and Williams(3) reported 87% excretion into bile 4 hours after an intravenous dose of 50 $\mu$g of cortisol-4-$C^{14}$. Although both groups have demonstrated an active enterohepatic circulation, this would not appear to be an important factor within 60 min. after injection, in view of the low rate of reabsorption of biliary radiometabolites after intragastric readministration(3).

Corticosterone has been shown to be the predominant corticoid in the rat. Fluctuation of endogenous adrenal output of corticosterone did not seem to be a major influence (Table IV). The amounts excreted during 60 min. in the adrenalectomized animals either with or without added corticosterone were in the same range as in the intact animals.

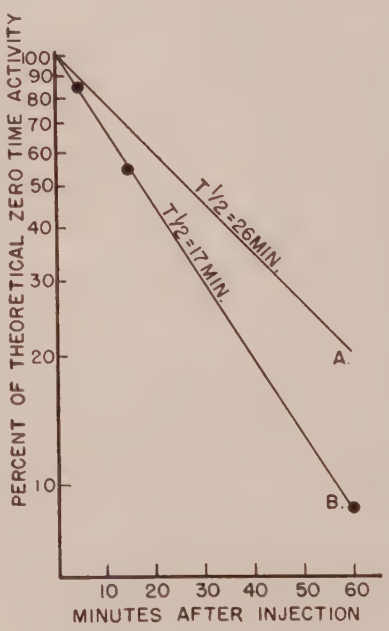


FIG. 2. A = Plasma radioactivity after inj. of 50 $\mu$g of cortisol-4-$C^{14}$ into external jugular vein, after Ulrich & Long(2). B = Plasma radioactivity after inj. of 14.5 $\mu$g of cortisol-4-$C^{14}$ into heart(4).

§ Schering Corp., Bloomfield, N. J.

TABLE IV. Distribution of Radioactivity, in % of Total Counts Injected, 60 Min. after External Jugular Vein Injection of Cortisol-4-C$^{14}$ (Alone or with Corticosterone) into Adrenalectomized Rats.

| Rat No. | 1 | 2 | 3 | Avg | 1 | 2 |
|---|---|---|---|---|---|---|
| Wt, g | 286 | 297 | 297 | 293 | 308 | 280 |
| Cortisol-4-C$^{14}$, $\mu$g | 12.0 | 12.0 | 12.0 | 12.0 | 8.5 | 8.5 |
| " , epm | 47,700 | 47,700 | 47,700 | 47,700 | 34,000 | 34,000 |
| Corticosterone, $\mu$g | | | | | 194 | 194 |
| GI tract & excreta | 39.9 | 37.8 | 51.0 | 42.9 | 47.4 | 47.4 |
| Liver | 11.7 | 11.9 | 8.7 | 10.8 | 8.1 | 10.6 |
| Kidneys | 3.2 | 2.3 | 2.4 | 2.6 | 1.6 | 1.9 |
| Plasma | 3.3 | 3.1 | 2.7 | 3.0 | 1.6 | 1.5 |

The liver is the main site of metabolism of cortisol in mammals, and in the rat it is also the main excretory organ. Englert and coworkers(8) have clearly shown that rate of disappearance of infused cortisol from the plasma of dogs is proportional to hepatic blood flow. In our experiments more rapid excretion of radioactivity after intracardiac injection was probably due to an acceleration of hepatic blood flow during the 1 hr after the puncture. A simultaneous decrease in blood flow through the skin could explain the low initial content in that organ. Such circulatory changes could well be produced by neural or endocrine reactions to the injection puncture. Cardiac puncture is a traumatic procedure undoubtedly causing prompt vasomotor changes and leaving injury to an extremely sensitive tissue well beyond the experimental period with unknown changes in the rates and patterns of arterial blood distribution. Manipulation of the jugular vein as it is dissected free as well as the venipuncture have frequently caused marked local venospasm. The tail vein injection, on the other hand, would appear to be the most innocuous route and should cause the least changes in circulatory dynamics. Patterns of distribution and slower rates of excretion after the intravenous injection would, therefore, more closely approximate the fate of endogenous hormone.

In view of these findings, previous studies on clearance of injected steroids should be interpreted in the light of possible hemodynamic changes caused by experimental conditions or manipulations and in such studies these changes should be controlled or measured, especially when using small animals.

*Summary.* Rate of excretion of radioactivity by rats was more rapid after intracardiac injection of cortisol-4-C$^{14}$ than after intravenous injection. In addition, after the latter route significantly larger quantities of radioactive material entered the skin immediately following injection. The most likely explanation for this difference is considered to be hemodynamic changes caused by heart puncture. The tail vein is the most suitable site of intravascular injection for studies of the fate of adrenal steroids in normal animals under basal conditions.

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# Modified Method for Measuring Potentiation of Barbiturate Anesthesia and its Relationship to Hypothermia. (24160)

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Various phenothiazine drugs with important effects on the central nervous system prolong duration of sleep caused by anesthetic doses of barbiturates(1), and subsequently this property of potentiation has been used as a basis for comparison of the relative central depressing activity of derivatives of phenothiazine(2). The phenothiazine depressant drugs also cause hypothermia in mice and a possible correlation between the properties causing hypothermia and prolongation of anesthesia has been reported, the inference being that hypothermia retards metabolism of the barbiturate and thereby prolongs duration of sleeping time(3). In the test usually used for measuring prolongation of sleeping time (o.c.), the mice lie in a heavily sedated state. If phenothiazine derivatives cause a fall in body temperature by affecting the central thermo-regulatory system, the marked inactivity of barbiturate anesthesia at room temperature will augment this fall. The same dose of phenothiazine alone, although reducing activity, will not produce the total inactivity of anesthesia. Thus in measurement of a correlation between hypothermia, where only the phenothiazine is given, and potentiation of barbiturates where both a phenothiazine and a barbiturate are given there are various interacting variables which have not been isolated and measured. We have developed a modified test for measurement of barbiturate potentiation which lends itself to investigation of the possible relationship mentioned above. The phenothiazine used in these tests was Win 13,645-5 (8-{3-[10-2-chlorophenothiazinyl)]propyl}-3αhydroxynortropane ethanesulphonate.

*Methods.* Potentiation of barbiturate sedation was determined in male albino mice of 18-22 g weight. Groups of 10 mice were pretreated with Win 13,645-5 subcutaneously or intravenously and after a chosen time interval (40 minutes is used in screening procedures) hexobarbital sodium was administered at a dose of 40 mg/kg intraperitoneally or 25 mg/kg intravenously (these will be called the intraperitoneal or intravenous subhypnotic doses, respectively). When loss of righting reflex occurred it did so within 15 minutes of administration of the sodium hexobarbital; the righting reflex was considered to be lost if the mouse when placed on its back did not regain its normal posture within 60 seconds. This test is of the "all-or-none" type and by using 3 graded doses of the phenothiazine derivative the $ED_{50}$ and its standard error can be calculated from log-probit plot(4). Groups of 20 mice were injected subcutaneously with the phenothiazine depressant drug, and rectal temperatures measured at chosen intervals by a standardized thermocouple device.* At temperatures of about 38°C this method gave readings with standard errors of $\pm 0.15$°C. The average rectal temperature of each group was calculated and compared with that of a control group.

*Results.* The dose-response relationship of sodium hexobarbital administered intraperitoneally and intravenously was determined. Doses of 40 mg/kg i.p. and 25 mg/kg i.v. were chosen as doses which when given alone, did not cause loss of righting reflex in mice; these doses henceforth will be called the i.p. and i.v. subhypnotic doses respectively.

Win 13,645-5 when administered at a dose of 20 mg/kg s.c. did not itself cause loss of righting reflex. However, pretreatment with Win 13,645-5 and other phenothiazine derivatives sensitized the mice so that administration of the i.p. subhypnotic dose of sodium hexobarbital caused loss of righting reflex, the $ED_{50}$ for Win 13,645-5 was $1.85 \pm 0.39$ mg/kg when the interval between administration of the 2 drugs was 40 minutes.

*Duration of action of Win 13,645-5.* The $ED_{50}$ will change according to the interval be-

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TABLE I. Effect of Lengthening Time Interval between Subcutaneous Premedication with Win 13, 645-5 and Administration of Intraperitoneal Subhypnotic Dose of Sodium Hexobarbital (30 Mice per Dose).

| Dose of Win 13, 645-5, mg/kg subcut. | Hours between medications | No. of mice losing righting reflex | $ED_{50} \pm$ S.E., mg/kg |
|---|---|---|---|
| 1.0 | 1 | 10 | 1.3 ± .14 |
| 1.6 | | 19 | |
| 2.5 | | 25 | |
| 1.2 | 2 | 7 | 1.65 ± .17 |
| 2.0 | | 20 | |
| 3.2 | | 28 | |
| 1.6 | 4 | 3 | 2.75 ± .31 |
| 2.5 | | 14 | |
| 4.0 | | 23 | |
| 2.5 | 6 | 7 | 3.7 ± .45 |
| 4.0 | | 15 | |
| 6.3 | | 26 | |

tween premedication with the phenothiazine derivative and administration of the barbiturate. Some estimate of duration of action can be made by varying this interval. Table I shows data obtained in such an experiment. The intervals between the 2 medications were 1, 2, 4, and 6 hours and the resulting $ED_{50}$ for each period being 1.3 ± 0.14, 1.65 ± 0.17, 2.75 ± 0.31 and 3.7 ± 0.45 mg/kg, respectively, representing equipotent doses at these intervals. Examination of the results in Table I shows that subcutaneous administration of 2.5 mg/kg caused 25 of the 30 mice to lose their righting reflex after one hour and after 6 hours was still present in sufficient quantity to cause 7 of the 30 mice to lose their righting reflex.

*Hypothermia.* The effects of the above equipotent doses on rectal temperatures of groups of 20 mice at 1, 2, 4, and 6 hours were determined. The relevant data from these experiments are given in Table II. Except at the 6 hour interval, average reductions in body temperature produced by equipotent doses are not significantly different, therefore doses found to cause the same degree of potentiation 1, 2, and 4 hours after administration also cause falls in body temperature which are not significantly different at 1, 2, and 4 hours after administration. The results indicate that both properties are produced by the same doses of Win 13,645-5 and that these experiments cannot separate the two activities.

Two further experiments were carried out in an attempt to separate these properties. In the first experiment the mice were pretreated with Win 13,645-5 intravenously and the i.p. subhypnotic dose of hexobarbital administered 5 minutes later. The results are presented in Table III. The $ED_{50}$ of 0.56 ± 0.10 mg/kg calculated from the results indicates a high order of activity in potentiation of sodium hexobarbital within 10 minutes of intravenous administration of Win 13,645-5. Although the total time between intravenous administration of Win 13,645-5 and loss of righting reflex was never more than 10 minutes, there is still the possibility of a loss of body temperature occurring during that time. Rectal temperatures were therefore taken immediately before and 10 minutes after intravenous administration of the same doses of Win 13,645-5. Resulting changes in body temperature were variable, average body temperature actually being higher 10 minutes after administration of phenothiazine than it was before, possibly due to repeated manipulation.

The second experiment was to administer Win 13,645-5 intravenously followed a few seconds later by the i.v. subhypnotic dose of sodium hexobarbital. These results (Table III) demonstrate a marked potentiation of sodium hexobarbital within a period of 2

TABLE II. Effect of Win 13, 645-5 on Rectal Temperatures of Mice (20 Mice/Dose).

| Dose,* mg/kg subcut. | Hours after medication | Avg rectal temp. ± S.E., °C | Avg rectal temp. of controls, °C | Fall in rectal temp. ± S.E., °C |
|---|---|---|---|---|
| 1.3 | 1 | 36.2 ± .2 | 36.9 ± .1 | .7 ± .2 |
| 1.7 | 2 | 36.4 ± .2 | 36.8 ± .1 | .4 ± .1 |
| 2.8 | 4 | 37.1 ± .2 | 37.8 ± .1 | .7 ± .2 |
| 3.7 | 6 | 36.1 ± .2 | 37.8 ± .1 | 1.7 ± .2 |

* These doses produced 50% potentiation of sodium hexobarbital at 1, 2, 4 and 6 hours, respectively.

TABLE III. Potentiation of Sodium Hexobarbital by Win 13, 645-5 Administered Intrav. (10 Mice/Dose).

| Win 13, 645-5 dose, mg/kg intrav. | Sodium hexobarbital | | No. of mice losing righting reflex | $ED_{50} \pm$ S.E., mg/kg |
|---|---|---|---|---|
| | Dose, mg/kg | Route | | |
| .32 | 40 | Intraper. | 1 | .56 ± .10 |
| .50 | " | " | 4 | |
| .80 | " | " | 8 | |
| .10 | 25 | Intrav. | 8 | |
| .20 | " | " | 9 | |
| Saline | " | " | 0 | |

minutes, before there could be any fall in body temperature. A control group given saline instead of Win 13,645-5 showed no potentiation of the i.v. subhypnotic dose of sodium hexobarbital.

*Discussion.* It is often difficult to separate 2 properties possessed by a drug unless they are produced at different dose levels. In the case of Win 13,645-5, reduction in body temperature of mice and potentiation of the sedative action of sodium hexobarbital both occur at the same dose levels. The fact that this is so does not necessarily mean that one property is the cause of the other. In the procedure used by others where large anesthetic doses of the barbiturate are given, and potentiation determined by measuring prolongation of anesthesia it is quite possible that the concurrent hypothermia will retard metabolism of the barbiturate. The fact that potentiation of sodium hexobarbital can be demonstrated within 2 minutes of intravenous administration of Win 13,645-5 indicates that this potentiation is independent of hypothermia.

*Summary.* A modified test for measurement of the property of a phenothiazine derivative to potentiate the action of barbiturates has been described. Using this technic, it has been shown that although there is some correlation between degree of hypothermia produced and potency of such a derivative to potentiate barbiturate sedation, these pharmacologic properties are primarily independent of each other.

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## Pre-staining Procedure for Electrophoretic Study of Serum Lipoproteins.* (24161)

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McDonald(1) introduced a new staining procedure for serum lipoproteins which involved staining of lipids before applying serum to paper for electrophoretic separation. This procedure as modified in our laboratory largely overcomes some of the difficulties encountered in previous studies of electrophoretic separation of $\alpha$ and $\beta$ lipoproteins utilizing post-staining procedures. Advan-

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tages of the pre-staining procedure include: conservation of time and reagents by omitting staining and rinsing of paper strips, better contrast between stained lipoprotein bands and unstained paper background, and also elimination of objection that rinsing solutions capable of washing dye from paper background may extract dye from lipoprotein bands as well. This paper presents a detailed study comparing the modified pre-staining procedure with the post-staining procedure for separation of serum lipoproteins by paper electrophoresis. It demonstrates advantages of the pre-staining procedure, and also presents clinical application of this procedure in a study of lipoprotein patterns in persons of differing age and sex.

*Method.* Acetylated Sudan black B was prepared as described by Lillie and Burtner (2). Occasionally a preparation failed to stain the lipids with any greater intensity than the nonacetylated product. When this occurred, the dye was discarded and a new batch prepared. Successful preparations of acetylated Sudan black B prepared from the same or different lots of Sudan black B showed no difference in lipid-staining properties. Five to 10 ml blood samples, taken in the postabsorptive state, were drawn into a clean, dry syringe; the needle was removed to prevent hemolysis and the sample carefully transferred to a glass test tube. After the clot was well formed, the sample was centrifuged in a refrigerated centrifuge at 5°C for 5 minutes at 2000 rpm. The serum was transferred to another tube and a saturated solution of acetylated Sudan black B (excess added to 100% alcohol, heated to 70°C for 10-15 minutes and filtered) was added with stirring, maintaining a serum-to-dye ratio of 10:1. After an hour the stained sample was centrifuged to remove excess dye particles. Twenty-five microliters of the stained serum were applied by means of a Spinco stripper to Whatman 3 MM filter paper strips on a Spinco Durrum-type electrophoresis cell containing veronal buffer (pH 8.6, 0.075 ionic strength) and a potential of 200 v was applied for 75 minutes. The strips were dried and scanned by means of a Spinco Analytrol. The results were expressed as $\beta/\alpha$ ratio, obtained by dividing the area under the $\beta$-lipoprotein curve by the area under the $\alpha$-lipoprotein curve. Since changes in the $\beta$ and $\alpha$-lipoprotein values usually appear to be reciprocal, this type of measure is more sensitive than percentage values. To compare pre- and post-staining technics, one serum sample was divided into 24 aliquots. Twelve of these aliquots were electrophoresed and stained according to the method of Swahn (3). The remaining 12 were prestained and electrophoresed according to the method here described. To test the reproducibility of this pre-staining procedure and the subsequent electrophoretic separation of lipoproteins, 6 serum samples were studied. Each was divided into 9 aliquots. After staining each aliquot, 8 strips were prepared for electrophoresis from 1 aliquot and electrophoresed on separate runs. One strip was prepared from each of the remaining 8 aliquots and electrophoresed in the same cell. In a study of the lipoprotein values with respect to age and sex, the lipoprotein patterns of 4 groups of subjects were determined over a 4-week period. The 4 groups included: young men, young women, older men, and older women. The young men were medical students; the young women, nursing students; and the older men and women were selected from geriatric wards of the San Bernardino County Hospital. All of the participating subjects maintained their usual pattern of living without major changes in activity or diet during the course of the experiment.

*Results.* An example of typical strips obtained from pre- and post-staining procedures is shown in Fig. 1. It will be seen that the patterns are similar, but the lipoprotein bands from the pre-stained serum stand out more distinctly and the densitometer curve is sharper. Data in Table I indicate that reproducibility of the pre-staining procedure is better than with our former practice of staining lipoproteins after electrophoretic separation on paper.

Comparison of $\beta/\alpha$ lipoprotein ratios of 8 strips from one pre-stained aliquot with ratios of the 8 strips from individually prestained aliquots showed no significant dif-

TABLE I. Lipoprotein Ratio as Determined by Pre-staining and Post-staining a Single Serum Sample. 12 strips/series.

| | Mean | S.E. | "T" | "P" |
|---|---|---|---|---|
| Pre-staining | 3.97 | .07 | | |
| Post-staining | 4.51 | .26 | 2.01 | 0.1 |

ference (Table II). This indicates that the procedure is reproducible both in electrophoretic separation and in staining of lipoproteins.

The mean $\beta/\alpha$ lipoprotein ratio for the group of young women was significantly lower than the ratios for each of the other 3 groups ($P<0.001$). Older women had a significantly lower ratio than older men ($P<0.05$). Young men also had a somewhat lower ratio than older men ($P<0.1$). A summary of these data is shown in Table III. These results indicate differences between the groups as measurements of cholesterol and lipoproteins by other investigators using different technics have shown(4-9).

TABLE II. Summary of Reproducibility Study of Pre-staining Procedure for Serum Lipoproteins.

| Sample | Mean values, $\beta/\alpha$ ratio | S.D. | S.E. | t |
|---|---|---|---|---|
| 1. a | 6.21 | .53 | .04 | 1.64 |
| b | 6.61 | .43 | .16 | |
| 2. a | 2.77 | .19 | .07 | .69 |
| b | 2.69 | .26 | .10 | |
| 3. a | 2.71 | .23 | .08 | .82 |
| b | 2.87 | .50 | .18 | |
| 4. a | 2.50 | .24 | .09 | .73 |
| b | 2.42 | .20 | .07 | |
| 5. a | 2.14 | .16 | .06 | .80 |
| b | 2.13 | .32 | .11 | |
| 6. a | 2.30 | .15 | .05 | .98 |
| b | 2.38 | .22 | .08 | |

a—8 strips, all from one pre-stained aliquot.
b—8 strips, one from each of 8 individually pre-stained aliquots.

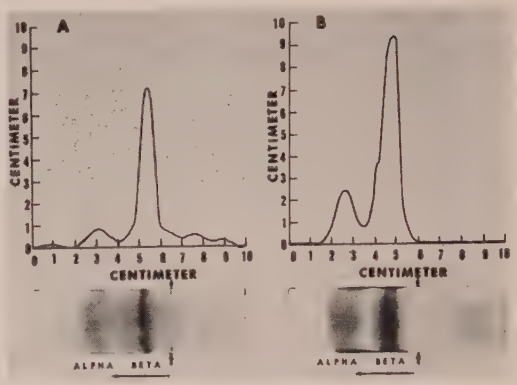


FIG. 1. Typical paper electrophoretic patterns and electrophoretograms of serum lipoproteins post-stained (A) with Sudan black B and pre-stained (B) with acetylated Sudan black B. Small arrows indicate point of serum application.

An expected difference in lipoprotein values is evident within the different groups and the values of individual subjects varied some from week to week. Such changes should be kept in mind where one attempts to relate a pathological condition to minor changes in the lipoprotein pattern.

In attempting to account for some of the changes seen in $\beta/\alpha$ lipoprotein ratios, it was found that the elevated values in the medical student group during the second and third weeks of observation coincided with a series of examinations. The possibility of a relationship between stress and lipid levels has been investigated further and some preliminary data have been reported(10).

*Summary.* A technic for staining serum lipoproteins prior to electrophoretic separation is reported. This procedure results in better reproducibility than is derived in the usual practice of staining paper strips after electrophoresis and also conserves on time and cost. When serial determinations are made with this procedure, in persons of differing age and sex, the lipoprotein ratios show differences be-

TABLE III. Summary of Differences in the $\beta/\alpha$ Lipoprotein Grouped According to Age and Sex.

| Subjects | Mean age | Wk 1 | Wk 2 | Wk 3 | Wk 4 | 4 wk avg | Area avg‡ $\alpha$ | Area avg‡ $\beta$ |
|---|---|---|---|---|---|---|---|---|
| Young women (16)* | 20 | 1.68 ± .11† | 2.29 ± .12 | 1.93 ± .93 | 1.95 ± .12 | 1.96 ± .11 | 30.1 | 55.9 |
| Older women (8) | 84 | 2.87 ± .32 | 3.38 ± .21 | 2.83 ± .09 | 3.56 ± .29 | 3.16 ± .23 | 23.5 | 65.8 |
| Young men (19) | 25 | 2.94 ± .18 | 3.77 ± .16 | 3.48 ± .17 | 3.05 ± .24 | 3.31 ± .19 | 17.6 | 51.5 |
| Older men (12) | 74 | 3.44 ± .21 | 3.33 ± .22 | 4.54 ± .26 | 4.01 ± .20 | 3.83 ± .22 | 23.8 | 81.9 |

* No. of subjects. † Mean ± S.E. ‡ Avg area under peaks of $\alpha$ and $\beta$ lipoproteins ($mm^2$).

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## Temperature-Sensitive Requirement for Methionine or p-Aminobenzoic Acid in Strain of *Escherichia coli*.* (24162)

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It has been known for at least 50 years(1) that naturally occurring strains of *Escherichia coli* can be cultured readily in simple synthetic media lacking specific growth factors. A simple source of carbon and energy such as glucose, glycerol or lactic acid and inorganic ammonium salts as sole source of nitrogen suffice for growth of the majority of *E. coli* strains. Occasional strains have been isolated, unable to grow in a minimal salts-glucose medium(2). This report deals with a naturally occurring strain of *E. coli* which behaves like the species prototype when grown at room temperature (21-24°C), but will not grow at 37°C unless the medium is supplemented with either methionine or p-aminobenzoic acid (PABA). This anomalous behavior is apparently associated with an abnormal reaction whereby homocysteine interferes with the role of PABA in synthesis of methionine.

*Materials and methods. Escherichia coli,* strain WM-13, was one of several strains isolated from clinical material at the Hospital for Women's Medical College of Pennsylvania and obtained through the courtesy of the late Dr. L. J. Kimmelman. It was identified as *E. coli* on the basis of morphology, lactose fermentation and typical IMViC reactions. It is susceptible to the action of coliphages T1, T3, T6 and T7, but resistant to T2, T4 and T5. It serves as sensitive indicator strain for temperate phages *lambda* and *w2*(3), and can be lysogenized by these phages. Growth requirement is unchanged by lysogenization. When lysogenized with *lambda* phage it loses its sensitivity to lysis by T1 and T7 and production of mature *lambda* phage can be induced with ultraviolet irradiation or with L-azaserine. Growth analyses were carried out in the salts-glucose (S-G) medium of Gots and Chu(4). The medium was inoculated to final concentration of $10^5$ cells/ml with a bacterial suspension harvested from overnight culture in nutrient broth and washed twice by centrifugation with minimal S-G media. Growth was recorded by turbidimetric readings with a Klett-Summerson photoelectric colorimeter equipped with green filter (#54).

*Results.* When incubation was carried out at 37°C, strain WM-13 was unable to grow in unsupplemented S-G medium. When medium was supplemented with either casein hydrolysate (0.1%) or with a mixture of B vitamins, even greater growth was obtained than in nutrient broth. Addition of single amino

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acids immediately revealed a specific growth requirement for methionine. Effective, though incomplete, restoration of growth was also obtained with leucine and α-aminobutyric acid. The effect of the B vitamin mixture could be completely replaced by the single addition of p-aminobenzoic acid (PABA).

To rule out the possibility that the alternate response to methionine or PABA might be due to a mixed population, tests for homogeneity of population were made by colony counts on solid media. The same number of colonies was obtained when the culture was plated on nutrient agar or on minimal agar containing either methionine or PABA. Unsupplemented controls showed no colonies even at inoculum size of $10^8$ bacteria/plate. When these plates were kept at room temperature for 24 hours colonies appeared on the unsupplemented plates of same size and number as those which had grown on supplemented plates at 37°C. Ability of strain WM-13 to grow in the unsupplemented S-G medium at room temperatures was readily confirmed in liquid media. Summary of the nutritional behavior of this strain at the 2 temperatures is given in Table I. At room temperatures strain WM-13 is indistinguishable from the average non-exacting *E. coli* strains usually encountered; only at 37°C is its alternate requirement for methionine or PABA manifested.

Table I also shows inability of known precursors of methionine to substitute for methionine. This indicates that the requirement for methionine is associated with a metabolic disturbance at the terminal conversion of homocysteine to methionine. Homocysteine, and to a lesser extent, homoserine proved inhibitory to normal growth at room temperature (Table I). Homocystine and homocysteine thiolactone were as effective as homocysteine in this inhibition.

TABLE I. Growth Response of *Escherichia coli*, Strain WM-13.

| Additions | μg/ml | Growth 37°C | Growth 21-24°C |
|---|---|---|---|
| None | 0 | 0 | 120 |
| DL-methionine | 20 | 105 | 103 |
| L - " | 20 | 103 | 102 |
| D - " | 20 | 45 | 112 |
| L-cysteine | 20 | 0 | 119 |
| DL-homoserine | 20 | 0 | 66 |
| Cystathionine | 40 | 0 | 104 |
| DL-homocysteine | 20 | 0 | 0 |
| DL-homocysteine thiolactone | 20 | 0 | 0 |
| DL-homocystine | 20 | 0 | 0 |
| Vit. $B_{12}$ | .1 | 0 | 116 |
| p-aminobenzoic acid | .01 | 109 | 113 |
| DL-leucine | 10 | 65 | 102 |
| Alpha-aminobutyric acid | 10 | 82 | 107 |

Growth is recorded as turbidity readings after 24 hr.

TABLE II. Reversal of Homocysteine Inhibition by Methionine.

| DL-homocysteine (μg/ml) | Growth (turbidity) DL-methionine None | DL-methionine 20 μg/ml |
|---|---|---|
| .0 | 160 | 160 |
| 12.5 | 5 | 150 |
| 25.0 | 0 | 155 |
| 50.0 | 0 | 135 |

Growth is recorded as turbidity readings after 24 hr at room temperature.

Inhibition by homocysteine was completely nullified by addition of methionine (Table II). Thus, homocysteine imposed a growth requirement at room temperature, which mimicked growth requirement at 37°C. PABA was also able to provide growth in the presence of homocysteine but, unlike methionine, activity of PABA depended on concentration of homocysteine. In the case of methionine, as long as the minimal amount necessary for optimal growth (20 μg/ml) was present, homocysteine was inactive over a wide range of concentrations. With PABA, the minimal amount for optimal growth at 37°C was 0.005 μg/ml. Growth in the presence of 50 μg of homocysteine/ml could not be obtained at room temperature until the concentration of PABA reached 0.1 μg/ml. Further increase of PABA to 10 μg/ml was required to prevent inhibition by 200 μg homocysteine/ml. Though this suggests a competitive type of relationship between PABA and homocysteine, a frank quantitative demonstration of such a relationship could not be obtained.

When a washed suspension of WM-13 cells was plated on unsupplemented media, derivatives (about $3/10^8$ cells) which no longer required methionine could be isolated after 3-4 days incubation at 37°C. These isolates were resistant to inhibition by homocysteine. Similarly, derivatives isolated from survivors of

homocysteine inhibition at room temperature no longer required methionine for growth at 37°C. This indicates that the methionine requirement at 37°C and inhibition by homocysteine at room temperature are alternate manifestations of a common metabolic lesion and that the lesion may be corrected by mutation. The mutant so obtained behaves like the average *E. coli* prototype.

*Discussion.* The unique nutritional behavior of strain WM-13 appears to be focused around a metabolic disorder in that function of PABA which is concerned with synthesis of methionine from homocysteine. Complete derangement of PABA metabolism is unlikely since formation of other end-products of PABA function (*e.g.*, purines, thymine, serine, etc.) remains intact. Ability of methionine alone to replace completely a requirement for PABA has previously been shown with mutants of neurospora(5-6) and under conditions of inhibition by 2-chloro-4-aminobenzoic acid(7). It is known that the synthesis of methionine is the first reaction to become limiting when PABA function is even partially inhibited(8-9).

The patterns of antagonism exerted by methionine and PABA on inhibition by homocysteine suggest that homocysteine, or a product derived from homocysteine, interferes with a cofactor (CoF) derived from PABA and that this interference, even if partial, prevents formation of methionine. This would create an anomalous and chaotic situation whereby homocysteine could prevent its own conversion to methionine. Isolation of homocysteine-resistant mutants which no longer require methionine indicates that inhibition by homocysteine and requirement for methionine are alternate manifestations of a common metabolic disorder. The temperature sensitivity of the requirement might well be due to an acceleration of metabolic activities which could lead to increased formation of endogenous homocysteine or increased conversion of homocysteine to an active inhibitor. Since homocysteine is not inhibitory to the average *E. coli* strain, the anomaly of strain WM-13 may be due to the possession of a deleterious reaction whereby homocysteine is diverted from its role as a precursor of methionine to the production of the inhibitor. Such a reaction would be either normally absent in the *E. coli* prototype or under suppressive control by a regulatory device. A mutation in WM-13 which would lead to the loss of the homocysteine by-pass would restore the typical *E. coli* growth pattern by allowing an economical utilization of homocysteine.

Both leucine and α-aminobutyric acid are structural analogues of homocysteine. As such, they could act as antimetabolites in activation of homocysteine and thus prevent formation of the inhibitor and conserve endogenous homocysteine for methionine formation. This would explain the ability of leucine and α-aminobutyric acid to support growth.

*Summary.* A strain of *Escherichia coli* (strain WM-13), isolated from clinical material, was unlike the average wild-type strains of *E. coli* in that it could not grow in a synthetic salts-glucose medium. Growth could be obtained only by supplementing the medium with either methionine or p-aminobenzoic acid. Except for partial stimulation by leucine and α-aminobutyric acid, none of the other naturally occurring amino acids or B vitamins could support growth. Growth requirement was evident only when cultivation was carried out at 37°C. At room temperature (21-24°C) full growth was obtained in the unsupplemented minimal medium, but addition of homocysteine again imposed a requirement for methionine or p-aminobenzoic acid. The temperature-sensitive growth requirement is apparently the result of a deleterious reaction, involving homocysteine as a substrate, which interferes with the function of p-aminobenzoic acid in the biosynthesis of methionine.

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# Hypoglycemic Potency in Man of a New Sulfonylurea Derivative (Chlorpropamide).* (24163)

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Previous studies have dealt with a comparison of carbutamide analogs, chlorpropamide and tolbutamide, with respect to hypoglycemic potency, as well as acute (P'an—personal communication) and chronic toxicities in mice, rats and/or dogs. The present studies were designed to compare the hypoglycemic potencies of chlorpropamide and tolbutamide in man at various times after single oral doses.

*Methods.* In the first experiment, 10 normal adult male subjects were studied who had been in a fasting state after 7:00 p. m. except for 8 ounces of milk at midnight. Venous blood glucose determinations were carried out using the method of Nelson(1) on specimens drawn at 7:00 a. m. Each subject then received by mouth either tolbutamide, 1 g., chlorpropamide, 1 g., or a placebo (sodium bicarbonate, 1 g). The subjects continued in fasting state on sedentary activity and blood glucose samples were drawn 1, 2, 3, 4 and 6 hours after each drug was administered. At weekly intervals, the tests were repeated on each subject using an alternate drug until each subject had received all 3 drugs. The order in which the drugs were administered was randomized, and the technician performing the tests did not know which drug had been administered. In a second experiment, 10 normal adult subjects who had supper at 5:00 p. m. received by mouth at 7:00 p. m. either tolbutamide, 1 g, chlorpropamide, 1 g, or sodium bicarbonate, 1 g. At 11:00 p. m. each subject drank 16 ounces of milk. Sedentary activity was permitted. At 7:00 a. m. the following day, 12 hours after the drugs were given, blood glucose samples were drawn, followed by a standard breakfast containing 52 g carbohydrate, 18 g of protein and 26 g of fat. At 1:00 p. m. (18 hours after the drug and 6 hours after the last feeding) another blood glucose was drawn. This procedure was repeated until each subject had been tested with each drug. The order of testing was randomized and the technician who performed the tests was unaware of which drug had been administered.

*Results.* Table I shows the mean changes in levels of blood glucose during a 6-hour period following administration of the 2 drugs and of the placebo. At 1, 2, 3, 4 and at 6 hours, mean blood glucose levels were significantly lower after chlorpropamide than after placebo ($p<.05$). On the other hand, after tolbutamide, values were significantly lower than placebo only from the second through the fourth hour. It may be seen that on the average, chlorpropamide produced greater hypoglycemic responses than tolbutamide at 1, 2, 3, 4 and 6 hours. At the third hour after medications, the differences between hypoglycemic responses to chlorpropamide and those seen with tolbutamide were statistically significant ($p<.05$, paired control t-test). The observation that chlorpropamide repeatedly led to lower blood glucose levels than did tolbutamide at all 5 periods of measurement lends weight to the evidence that the former does induce a greater hypoglycemia. This observation would be expected to occur by chance only once in 32 trials ($p<.05$).

Table II shows mean blood glucose levels of 10 subjects at 12 and 18 hours following administration of each drug and of the placebo. These data were analyzed using a paired controls t-test. The analyses indicated that a significant hypoglycemic effect was produced by chlorpropamide at 12 hours ($p<.02$) and at 18 hours ($p<.02$). On the other hand, the effect produced by tolbutamide was not statistically significant at 12 or at 18 hours although the mean blood glu-

* Chlorpropamide was supplied by Charles Pfizer and Co., as *Diabenese.*

TABLE I. Mean Changes (± S.D.) in Blood Glucose Levels after Placebo, Chlorpropamide and Tolbutamide in 10 Normal Subjects.

| Drug | | Control in mg % at 7 a.m. | 1 hr | 2 hr | 3 hr | 4 hr | 6 hr |
|---|---|---|---|---|---|---|---|
| $NaHCO_3$ | 1 g | 76 ± 7.9 | − 2 ± 3.9 | − 3 ± 4.0 | − 4 ± 6.3 | − 5 ± 6.8 | − 6 ± 7.0 |
| Chlorpropamide | " | 76 ± 5.6 | −12 ± 11.0 | −15 ± 5.3 | −17 ± 7.4 | −15 ± 8.4 | −12 ± 6.7 |
| Tolbutamide | " | 77 ± 4.7 | − 5 ± 8.2 | −11 ± 6.4 | −13 ± 6.3 | −10 ± 7.4 | −11 ± 7.3 |

See text for degree to which differences statistically significant.

cose values were slightly lower after tolbutamide than after the placebo at both 12 and 18 hours.

None of the subjects exhibited significant toxic effects from either chlorpropamide or tolbutamide, although in a few instances subjects reported symptoms which could have been on the basis of mild hypoglycemia, such as headache and tremulousness. In 2 instances these symptoms occurred 1 to 2 hours after chlorpropamide at which time the levels of blood glucose were less than 55 mg %.

*Discussion.* It is evident that chlorpropamide is a potent hypoglycemic agent. It is not possible on the basis of these data to define clearly its time-action curve. It has been shown that the blood levels of chlorpropamide 24 hours after single doses are greater than blood levels of tolbutamide after equivalent doses of tolbutamide(1). This suggests that the greater potency of chlorpropamide might be due to slower degradation or slower excretion of that drug. On the other hand, it does not seem likely that a slower rate of degradation or excretion could account entirely for the increased potency in man of chlorpropamide as compared to tolbutamide since differences in potency were impressive as early as 1 to 3 hours after administration of the drugs (Table I).

In the diabetic patients whom we have treated with daily doses of chlorpropamide, the fasting blood glucose level gradually declined during the first 4 to 7 days of treatment before stabilizing, suggesting that daily administration produced a cumulative effect. This suggests that the half-life of this drug is similar to that of carbutamide, which is approximately 33 hours(2). The biologic half-life of tolbutamide is approximately 4 hours(2).

The degree to which chlorpropamide will be useful in the treatment of diabetes will depend also, of course, on its toxicity in man which we are not yet able to evaluate adequately.

TABLE II. Mean Changes in Blood Glucose Levels of 10 Normal Subjects 12 and 18 Hr after Chlorpropamide and Tolbutamide.

| At 7 a.m., 12 hr after drug | | | At 1 p.m., 18 hr after drug | | |
|---|---|---|---|---|---|
| Control,* | Chlorp. | Tolb. | Control | Chlorp. | Tolb. |
| 73 ± 4.0† | −6 ± 4.7 | −2 ± 6.8 | 73 ± 3.6 | −5 ± 5.6 | −2 ± 7.0 |

* Blood glucose at 7 a.m., 12 hr after a placebo.
† Stand. dev.

See text for degree to which differences statistically significant.

*Summary and conclusions.* A single oral dose of 1 g of chlorpropamide produced a significant hypoglycemic effect in normal subjects at 1, 2, 3, 4, 6, 12 and 18 hours after administration by mouth. At each of these hours the hypoglycemic effect produced was greater than that produced in these subjects by an identical dose of tolbutamide.

We would like to acknowledge the technical assistance in these experiments of Charles Key, Bill J. Matter, B. H. Henry and Dorothy Antonia Wood.

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## Effects of Age and Mode of Ingestion on Absorption of Plutonium.*† (24164)

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Gastrointestinal absorption and distribution of plutonium in the rat has been studied in detail(1-3). In most experiments, absorption was determined for young adult animals. The gavage method of administration has been employed extensively since it permits accurate calibration of the dose delivered. From the standpoint of personnel hazard however, stomach intubation does not adequately represent the conditions of accidental oral ingestion where the oral cavity and esophagus may also be exposed to the radioisotope. Further studies on plutonium metabolism showing the influence of age and method of oral administration on absorption are presented here.

*Methods.* Rats were of the Sprague-Dawley strain, ranging in age from 1 to 84 days. The very young animals were gavage fed using a ¼ cc syringe fixed with a length of plastic intravenous tubing drawn out to about the diameter of a 27 gauge needle. Each rat received approximately 100 $\mu$g of plutonium by gavage in 50 $\mu$l of a pH 2 nitric acid solution. At least 5 rats were fed in each age group. After 21 days the rats were sacrificed and plutonium content determined in liver, lung, carcass and, in some animals, total bone and soft tissue. Lungs of all rats were analyzed to determine if exposure had been intratracheal rather than intragastric. The young were returned to the dams after feeding and maintained in the usual manner until sacrificed. Adult rats were used in the studies to show the effect of the method of oral administration on absorption. Those rats administered $Pu^{239}$ directly on the tongue received 1.4 $\mu$g of plutonium in 5 $\mu$l of either a 0.1 *N* or 2 *N* nitric acid solution. Animals were sacrificed after 7 days and the head, liver, and total skeleton analyzed for plutonium content. Rats fed plutonium in drinking water were exposed for 24 days to pH 2 water containing 66 $\mu$g of plutonium/liter. Previous studies showed percentage of the dose absorbed did not vary within a dose range of $10^{-5}$ $\mu$g/ml to 1 $\mu$g/ml(2). Daily consumption amounted to about 1 $\mu$g after correcting for loss due to dripping from water bottles. Samples taken at sacrifice included liver and skeleton.

*Results.* Gastrointestinal absorption of plutonium at several ages is shown in Fig. 1. In day-old rats absorption was 85 times that of the adult(2). The amount absorbed dropped abruptly in 21-day-old rats to near adult levels. The % absorbed varied considerably among animals in this group, possibly because of dietary and metabolic changes associated with this age of weaning. After age 30 days gastrointestinal absorption was not significantly different from the adult level, except for the unexplained high absorption found in the 56-day group. Plutonium distribution in the 7-day-old rat was essentially the same as that reported for the adult(2). Of the total plutonium retained, 80% was in the skeleton, 15% in the liver and 5% in the soft tissues.

Results of the study of plutonium absorption and distribution following oral administration are presented in Table I. Total absorption after exposure to the tongue was approximately 20 times lower than has been reported for similar solutions fed intragastrically(2). Holdup of plutonium in the mouth of these animals was still appreciable 7 days after exposure, especially for the more acid solution. On examination at sacrifice, the oral cavity showed no gross damage due to the 2 *N* acid. Absorption after drinking water containing plutonium for 24 days was in close agreement with results obtained from previous gavage feeding experiments(2).

*Discussion.* Conclusions based on extrapo-

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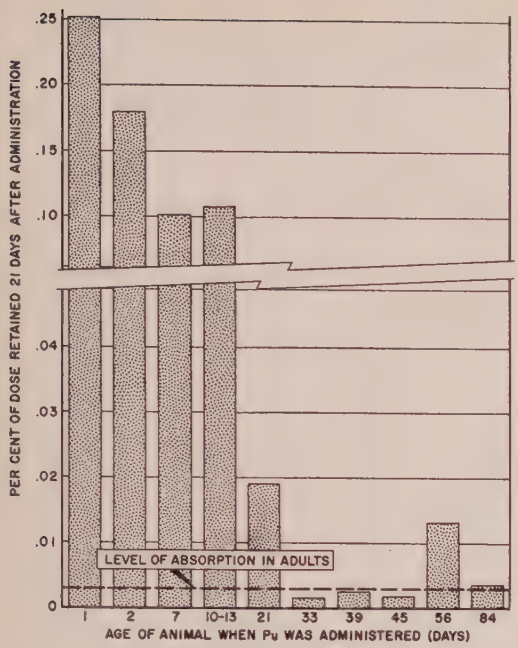


FIG. 1. Gastrointestinal absorption of plutonium by rats of different ages.

lation of plutonium absorption in the immature rat to any particular age in the human infant are of doubtful significance because of the uniquely long pre-pubertal period in man compared to other mammals. During this early development little correlation exists between man and rat in age-growth relationships by which some comparison might be made(4). However, these results do give a quantitative measure of an enhanced absorption that exists during early development and differentiation of the intestinal tissues. Of interest in this regard is the apparent permeability of the newborn animal intestine to some of the proteins present in the colostrum. Histological studies have shown globulins absorbed without change from the intestine of the calf 24 to 48 hours after birth(5). Enhanced absorption of plutonium by immature rats may be further evidence of this increased intestinal permeability to relatively complex materials. Of further interest is the observation that colostral protein is not absorbed appreciably by rats after age 21 days(6). Plutonium absorption at this age was found to be markedly decreased to near adult levels.

The only suggestion of an increased hazard from ingested plutonium due to exposure of the oral cavity and esophagus lies in the prolonged retention of an appreciable fraction of administered plutonium in the head. This was observed only following the application of solutions of high plutonium concentration directly on the tongue, and was not reflected in any increased absorption and deposition in bone. Under usual conditions of exposure it seems reasonable to conclude that exposure of the oral cavity and esophagus will lead to no increase in the hazard.

TABLE I. Absorption and Distribution of Orally Administered Plutonium (% of Administered Dose per Tissue).

| Tissue | Single dose on tongue | | 24 days in drinking water, pH 2 |
|---|---|---|---|
| | 2 N $HNO_3$ | .1 N $HNO_3$ | |
| Bone | .003 | .005 | .002 |
| Liver | .003 | .002 | .006 |
| Head | .4 | .08 | |

*Summary.* The effect of age on gastrointestinal absorption of plutonium was determined for rats over the range 1 to 84 days of age. Absorption for 1-day-old rats was 85 times that reported for the adult. This dropped to near adult levels in the 21-day-old weanlings. Plutonium distribution in the 7-day-old rat was essentially the same as that found for the adult. Absorption of plutonium applied directly to the tongue or fed in drinking water did not differ significantly from absorption following gavage feeding. It was concluded that the hazard of ingested plutonium is not increased when exposure of the oral cavity and esophagus is involved.

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# Serum Ribonuclease Activity After Temporary Ligation of Renal Pedicles in Rats.* (24165)

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The previously described increase in serum ribonuclease (*RNase*) activity after bilateral nephrectomy in several species(1) suggested investigation of the behavior of this enzymatic activity in renal failure(2) which follows temporary constriction of the renal pedicles.

It will be shown that the above procedure performed on rats leads to increases in serum *RNase* activity, reversible in surviving animals, and markedly paralleling blood urea levels.

*Material and methods.* Adult Wistar rats of both sexes were used. Under brief ether anesthesia both renal pedicles were ligated with thick thread. Forty-five, 60 or 90 minutes later the rats were reanesthetized and the ligatures taken off. No heparin(3) was used, as this substance inhibits serum *RNase* activity(4,5). After removal of ligatures, the kidneys returned to uniform pink color. Controls were handled similarly, but ligatures were not tied. Blood samples of approximately .3 ml were taken from tail tips after warming animals at 37°C for 10 minutes. Most animals were sampled immediately before and at end of constriction period, and at intervals up to 3 to 4 weeks after start of experiment. *RNase* activity was determined in 4 $\mu$l duplicate samples of serum as previously described(1,5), with the modification that 5% (w/v) trichloroacetic acid was added to the acetone-HCl precipitating mixture. The dialyzed substrate could be stored at −15°C for at least 6 months. Samples from each experiment were simultaneously assayed. Results are expressed in optical density units. Urea was determined in duplicate 20 $\mu$l serum samples by nesslerization after urease digestion(6).

*Results.* Mortalities in the 3 experimental groups, with 45, 60 and 90 min. constriction, were, 3/14 (totals of 2 experiments), 8/14 and 5/7, the one for the 60 min. group being significantly greater than Koletsky and Gustavson's data(3).

*Behavior of blood urea levels.* In surviving animals there was a rapid and reversible increase in serum urea, which agrees with the findings of other workers(3). In animals that did not survive, urea remained at high levels until death. The relatively small number of animals studied did not disclose relation between magnitude of urea rise and length of time the ligatures were applied. Table I shows results of one of the 45 min. experiments. The vertical lines indicate 95% confidence limits of the means. Analysis of variance of this experiment for 5 surviving† rats revealed significant ($P<.01$) effects of both "between rats" and "between times" factors. Comparisons between zero hour mean, and those of other time periods by the method of Scheffé(7), showed significantly ($P<.05$) increased urea levels up to 6 days. The 18 and 30 days group did not differ significantly from the zero hour group.

*Behavior of serum RNase* was similar to that of urea. No clear relationship was found between rise in enzyme activity and period of constriction on the one hand, or surviving-non-surviving condition on the other. In surviving animals enzymatic activity was reversibly increased, whereas in those which died, it remained at high levels. It should be noted that, in agreement with our previous experience, large variability was found in initial control values of different animals. Thus, a 4-fold range of values was found. When initial levels were compared with those immediately following release of ligatures, *RNase* activity was a more sensi-

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† Two dead animals and 2 surviving ones were excluded from the analysis because of missing values.

TABLE I. Serum Ribonuclease Activity and Urea Levels in 9 Rats before and after a 45 Min. Ligation of Renal Pedicles.

| | Control | 45 min. | 12 hr | 1 day | 2 days | 4 days | 6 days | 18 days | 30 days |
|---|---|---|---|---|---|---|---|---|---|
| R | 103 | Det. lost | 194 | 230 | 275 | 307 | 56 | 8 | 11 |
| U | 41 | 50 | 108 | 83 | 75 | 83 | 43 | 36 | 29 |
| R | 100 | 125 | 87 | 116 | 184 | 223 | 201 | 47 | 24 |
| U | 73 | 70 | 120 | 133 | 125 | 75 | 39 | 48 | 50 |
| R | 42 | 111 | 87 | 84 | 139 | 152 | 67 | 52 | 84 |
| U | 41 | 100 | 76 | 58 | 39 | 37 | 41 | 37 | 50 |
| R | 27 | 42 | 46 | 128 | 155 | 109 | 49 | 0 | 0 |
| U | 43 | 54 | 100 | 124 | 116 | 78 | 46 | 43 | 46 |
| R | 78 | 205 | 256 | 150 | 312* | | | | |
| U | 62 | 64 | 100 | 38 | 129 | | | | |
| R | 40 | 134 | 246 | 218* | | | | | |
| U | 66 | 66 | 158 | Det. lost | | | | | |
| R | 54 | 90 | 16 | 99 | 165 | 137 | 73 | 16 | 23 |
| U | 46 | 51 | 66 | 71 | 18 | 50 | 43 | 28 | 29 |
| R | 83 | 127 | 136 | 225 | 198 | 61 | 124 | 77 | 55 |
| U | 46 | 71 | 112 | 149 | 124 | 53 | 51 | 54 | 51 |
| R | 112 | 223 | 230 | 247 | 381 | 96 | 80 | 73 | 60 |
| U | 50 | 62 | 83 | 158 | 91 | Det. lost | 67 | 66 | 83 |
| R | 71.0 ± 10.4† | 132.1 ± 20.7 | 144.2 ± 30.1 | 166.3 ± 21.2 | 226.1 ± 30.5 | 155.0 ± 27.8 | 92.8 ± 20.2 | 39.0 ± 11.7 | 36.2 ± 11.4 |
| U | 52.0 ± 3.9 | 65.2 ± 4.9 | 102.5 ± 9.03 | 101.7 ± 15.8 | 81.1 ± 20.6 | 62.5 ± 7.4 | 47.3 ± 3.5 | 44.6 ± 4.7 | 48.2 ± 6.7 |

R = Ribonuclease activity (optical density × 1000). U = Urea, mg %.
* Indicates death of animal. † Mean ± stand. error.

tive indicator than urea of the exclusion of the kidneys. After 45 minutes of total ischemia, analysis of 8 paired values by the *t* test revealed a significant increase of about 85% in the *RNase* activity, whereas a similar analysis of the urea levels showed a 27% increase, which was not significant at the 5% probability level.

Results of the 45 minute constriction experiment are given in Table I. The large confidence limits of the means are due to the above reported large disparity between initial serum enzyme activities of different animals. Analysis of variance for 5 surviving animals† showed that the effects "between rats" and "between times" were significant ($P<.01$). Study of the contrasts between means of the zero hour group and those of the other time periods(7) showed significantly increased *RNase* activity levels up to 4 days; the others were not significant.

*Control animals.* Fourteen control rats were studied for varying periods. In 11 of them serum *RNase* and urea were either unchanged or decreased. In the 3 remaining animals erratic variations were observed. A sample of control rats is given in Table II.

*Discussion.* It is known that temporary constriction of artery or renal pedicles in the rat(8) and rabbit(9) leads mainly to necrosis of the proximal convoluted tubules. Functional studies in the dog(10-13), indicate that an immediate reduction in the glomerular filtration rate follows renal ischemia, accompanied by a decrease in secretory ability of the tubules and in renal blood flow. In surviving animals these phenomena are either partially or completely reversible. It can be assumed that similar functional impairment would also be found in the rat. It should be noted that the behavior of blood urea is similar in the rat(3), rabbit(9) and the dog(11, 14).

The rapid increase in serum *RNase* activity which follows exclusion of the kidneys(1) could be explained by the assumption that plasma *RNase* is efficiently filtered by kidney glomeruli, although alternative views could also be held. On the other hand, the clearance of the enzyme being only around 2% of

TABLE II. Serum Ribonuclease and Urea Levels in Control Animals.

| | Control | 24 hr | 36 hr | 48 hr | 4 days | 7 days |
|---|---|---|---|---|---|---|
| R | 73 | 43 | 53 | 42 | 27 | 40 |
| U | 48 | 37 | 42 | 23 | 42 | 42 |
| R | 88 | 62 | 52 | 44 | 114 | 104 |
| U | 40 | 29 | 29 | 33 | 21 | 25 |
| R | 82 | 18 | 34 | 31 | 92 | 45 |
| U | 42 | 37 | 33 | 25 | 32 | 50 |
| R | 244 | 185 | 190 | 191 | 166 | 196 |
| U | 27 | 30 | 21 | 36 | 39 | 37 |
| R | 122.0 ± 40.8* | 77 ± 37.1 | 82 ± 36.1 | 77 ± 38.1 | 100 ± 28.7 | 96 ± 36.2 |
| U | 39.3 ± 4.5 | 33.3 ± 2.1 | 31.2 ± 4.2 | 30.1 ± 2.1 | 33.5 ± 2.3 | 38.5 ± 5.1 |

R = Ribonuclease activity (optical density × 1000). U = Urea, mg %.
* Mean ± stand. error.

endogenous creatinine clearance in the rat(15, 16), a high tubular reabsorption with inactivation should be further postulated(17,18).

That the increase in serum *RNase* activity is not secondary to the uremic condition and that the kidney is involved in inactivation of the enzyme, is shown by unpublished observations on dogs on which ureter venous anastomosis was performed bilaterally. In these experiments the increase in serum *RNase* is much smaller than that of binephrectomized controls, while degree of uremia is similar in both cases.

We wish to suggest that the above reported increase in serum *RNase* activity after renal ischemia is mainly due to reduction in glomerular filtration rate. It cannot be excluded, however, that either production, or release of enzyme to the blood, or both, may be increased by our experimental procedure, or that backflow through the damaged tubules contributes towards elevation of serum enzyme activity. Nothing is known, however, of homogeneity and physical characteristics of serum *RNase*. Pending these and other physiological data, interpretation of our findings must be speculative. The above presented hypothesis has the only merit that it may be experimentally tested.

Our findings of the parallelism between behavior of serum urea and *RNase* activity in the rat suggested that this enzyme could be profitably studied in several experimental and clinical conditions. Enzyme assays performed on plasma from normal and uremic human subjects have yielded similar results, to be reported later.

*Summary.* A study was performed of urea and serum ribonuclease activity after ligation of renal pedicles in rats for periods of 45, 60 or 90 minutes. Both urea and ribonuclease activity increased reversibly in surviving animals and remained elevated until death in the others. The significance of the findings is discussed, and it is suggested that the decrease in glomerular filtration rate could explain the behavior of serum ribonuclease.

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## Effect of Different Intrinsic Factor Preparations on $Co^{60}$ Vit. $B_{12}$ Uptake in Rat Liver Slices. (24166)

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Miller and Hunter(1) reported that radioactive Vit. $B_{12}$ is absorbed by rat liver slice, and that this absorption is increased markedly by addition of intrinsic factor rich extracts of hog stomach. This work has been confirmed (2,3). That this increase in binding is related to intrinsic factor present in the concentrate is suggested by the fact that it is abolished by boiling of intrinsic factor concentrate(1), and that proteins without known intrinsic factor activity fail to produce this increased binding. These proteins include gelatin, lysozyme, crystalline pepsin and pink protein(1). Addition of human gastric juice failed to enhance Cobalt-60 labeled Vit. $B_{12}$ uptake by rat liver slice. Since this was similar to the results obtained with human gastric juice in gastrectomized rats, it seemed advisable to extend these observations to rat gastric juice, dog gastric juice, hog intrinsic factor concentrates and hog gastric juice to test the effect these intrinsic factor preparations have on liver slice technic.

*Technic.* Seventy-five to 100 mg slices were prepared from chilled rat livers and incubated for 30 min. at 37° in Warburg vessels containing Hastings' buffer(4) 15 m$\mu$g radioactive Vit. $B_{12}$ and measured amounts of gastric juice. Prior to incubation the system was flushed 5 min. with 5% $CO_2$ and 95% $O_2$ mixture. After incubation, the slices were washed in fresh buffer. We found that the number of washes or length of time used in washing does not cause a change in radioactivity absorbed. The slices were transferred to glass test tubes and assayed in scintillation well type counter. The counts were corrected for background and expressed as specific activity (counts/min./100 mg). Various amounts of rat, dog, human and hog gastric juice were incubated with the liver slices. Rat gastric juice was obtained by placing a ligature over pylorus of rat stomach. Eight hours later the gastric juice was collected. Gastric juice was collected from dogs with Heidenhain pouches which had been stimulated by raw meat. Human gastric juice was obtained from fasting patients by gastric tube after insulin stimulation. Hog gastric juice was obtained by expressing gastric juice from surface of freshly slaughtered hog stomach. The hog intrinsic factor concentrate was furnished us.* In patients with pernicious anemia, this intrinsic factor concentrate is active at a dosage of 2-3 mg. The binding ability of various gastric juice fractions as tested by the method of Gregory and Holdsworth(5), *viz.* 0.61 $\mu$g of Vit. $B_{12}$ containing 0.01 $\mu$c of $Co^{60}$ were added to 5 mg of gastric juice. This mixture was dialyzed against tap water for 48 hours and the contents of dialysis tubing counted in scintillation well counter. These gastric juices were tested in a patient with pernicious anemia to prove that each had intrinsic factor activity. Twenty-five mg of

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TABLE I. Effect of Gastric Juice on $Co^{60}$ Vit. $B_{12}$ Liver Uptake in a Patient with Pernicious Anemia.

| | |
|---|---|
| Control | .9% |
| 25 mg rat gastric juice | 3.5 |
| " dog " " | .4 |
| " human " " | 5.4 |
| " hog " " | 5.0 |

Normal value = >4.7%

.1 $\mu$c $Co^{60}$ .1 $\mu$g vit. $B_{12}$

gastric juice were added to 0.1 $\mu$g of $Co^{60}$ labeled Vit. $B_{12}$ and fed to a patient with pernicious anemia. The seventh day liver uptake of the $Co^{60}$ was obtained by placing a 5" plastic scintillation detector over the liver area. The radioactivity measured was compared to a standard and is reported as per cent uptake(6).

*Results.* Table I shows per cent liver uptake of $Co^{60}$ labeled Vit. $B_{12}$ in pernicious anemia. The control value without added intrinsic factor was 0.9%. A normal patient will have a liver uptake greater than 4.7%. Addition of hog, rat and human gastric juice, increased this patient's liver uptake of Vit. $B_{12}$, thus proving that these preparations exhibit intrinsic factor activity. Dog gastric juice resulted in no increase.

Table II shows relative binding abilities of these gastric juice fractions. The greatest binding was exhibited by hog intrinsic factor. Binding ability of hog and rat gastric juice was approximately equal.

Fig. 1 shows the results obtained with rat liver slice. The abscissa is in net counts/min 100 mg of rat liver. Ordinate represents increasing amounts of added gastric juice fractions. The uptake of $Co^{60}$ Vit. $B_{12}$ increase when 30 $\mu$g of hog intrinsic factor concentrate was added. Adding 100 $\mu$g concentrate decreased uptake to the control level and adding over 150 $\mu$g inhibited this uptake. These results are nearly identical to those obtained by Miller and Hunter(1). Hog gastric juice produced the same degree of enhancement of the liver uptake as did the hog intrinsic factor concentrate. However, this occurred after 150 $\mu$g of gastric juice had been added instead of the 30 $\mu$g that produced maximal uptake using hog intrinsic factor concentrate.

TABLE II. $Co^{60}$ Vit. $B_{12}$ Binding Ability of Gastric Juice.

| Source | $\mu$g |
|---|---|
| HIFC | .129 |
| Human | .044 |
| Dog | .030 |
| Hog | .016 |
| Rat | .015 |

$\mu$g non-dialyzable vit. $B_{12}$/mg gastric juice.

Dog gastric juice increased uptake to a greater degree than that produced by the hog intrinsic factor concentrate. At all concentrations, rat gastric juice slightly inhibited uptake of $Co^{60}$ Vit. $B_{12}$ by the rat liver slice.

Human gastric juice caused no enhancement of uptake. Inhibition was present even at the lowest concentrations, and increased with increasing dosages. Table III shows actual mean values obtained in these determinations.

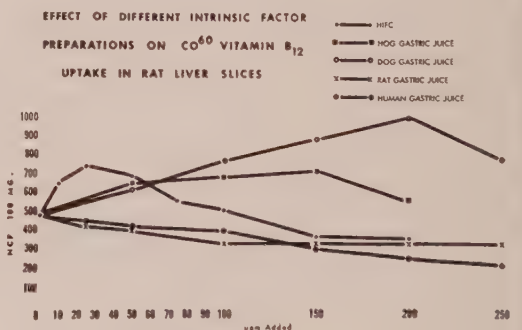


FIG. 1.

*Discussion.* With the exception of dog gastric juice, the gastric juices used in this experiment contained intrinsic factor since each one was capable of increasing Vit. $B_{12}$ absorption in patients with pernicious anemia. Dog gastric juice was collected from Heidenhain gastric pouches, made up of part of the fundus and the cardia of the dog stomach. By measuring fecal excretion of $Co^{60}$ Vit. $B_{12}$ we have found that 25 mg doses of dog Heidenhain pouch juice were active in one of 4 other pernicious anemia patients. These data do not prove presence or absence of intrinsic factor in the pouch juice, since it may not be active in the human.

However, the effect on a rat liver slice was quite different. Preparations from the dog and hog increased Vit. $B_{12}$ binding. Human

TABLE III. $Co^{60}$ Vit. $B_{12}$ Uptake by Rat Liver Slice.

| μg added | Hog conc. | Hog | Dog | Human | Rat |
|---|---|---|---|---|---|
| 10 | 641 ± 9* | | | | |
| 25 | 730 ± 19 | | | 474 ± 12 | 441 ± 14 |
| 50 | 656 ± 17 | 658 ± 19 | 640 ± 16 | 420 ± 9 | 425 ± 17 |
| 100 | 497 ± 16 | 687 ± 11 | 763 ± 7 | 393 ± 9 | 340 ± 20 |
| 150 | 361 ± 14 | 735 ± 11 | 888 ± 6 | 303 ± 13 | 333 ± 8 |
| 200 | 360 ± 14 | 558 ± 7 | 1018 ± 20 | 250 ± 16 | 342 ± 13 |
| 250 | | | 802 ± 25 | 216 ± 8 | 325 ± 22 |
| | Control 485 ± 19 | | Net counts/min./100 mg liver | | |

* Mean ± S.E. of mean.

gastric juice markedly inhibited while rat gastric juice only slightly inhibited the binding. If this method is actually measuring intrinsic factor activity, we must conclude that there is a species difference in the mechanism of action of intrinsic factor.

It is probable that the gastric juices used here have different concentration ratios of intrinsic factor and non-specific Vit. $B_{12}$ binding proteins. Three of the 4 gastric juices used were capable of correcting the specific absorptive defect in a patient with pernicious anemia and all 4 gastric juices would presumably correct the Vit. $B_{12}$ absorptive defect in gastrectomized animals of the same species. Therefore if the failure of human and rat gastric juice to enhance Vit. $B_{12}$ uptake by the rat liver slice were due to an excessive amount of non-specific Vit. $B_{12}$ binding protein, we must conclude that the liver slice is more sensitive to these ratios than is the gastrointestinal tract. That this may be true is suggested by the work of Herbert(3). By sequential incubation of the liver slice, he was able to show an increased uptake using human gastric juice from patients with Sprue and Vit. $B_{12}$ dietary deficiency states.

An alternate explanation for the findings obtained here is that liver slice uptake may not be related to intrinsic factor activity. It has long been known that gastric juice will bind Vit. $B_{12}$, making it non-dialyzable and unavailable to some micro-organisms. That this binding phenomenon is not synonymous with intrinsic factor has been confirmed(7,8). They have shown that degree of Vit. $B_{12}$ binding is not well correlated with intrinsic factor activity and that gastric fractions are obtainable that will bind Vit. $B_{12}$ but which have no intrinsic factor activity. Relative binding potency of the gastric fractions used in this experiment did not correlate with their intrinsic factor activity in a pernicious anemia patient or their uptake by the liver slice. Whether the binding of Vit. $B_{12}$ to proteins other than intrinsic factor is related to the uptake phenomenon obtainable with the liver slice is not known. Miller(9) has shown that binding of Vit. $B_{12}$ to human serum proteins is enhanced by addition of gastric juice obtained from normals and pernicious anemia patients who presumably have little intrinsic factor. There is a good possibility that these 2 reactions of gastric juice with serum proteins and with the liver slice may be similar, since each is inhibited by pseudo-Vit. $B_{12}$. If this is so, the rat liver slice technic is not measuring intrinsic factor activity but is measuring another type of binding of Vit. $B_{12}$ which, at the present time, is without known physiological significance.

*Summary.* (1) We have confirmed the work of Miller and Hunter and have shown that addition of hog intrinsic factor to rat liver slice will increase uptake of $Cobalt^{60}$ labeled Vit. $B_{12}$. (2) Gastric juices with intrinsic factor activity from other animal sources have different effects on this uptake and this is not correlated with intrinsic factor activity in the human or their ability to make Vit. $B_{12}$ non-dialyzable.

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## Tissue Distribution of $C^{14}$-Labeled Bacterial Polysaccharide in Guinea Pig, Rat, Mouse and Rabbit.*† (24167)

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In the guinea pig the adrenal gland has the highest tissue concentration of $C^{14}$ after intravenous injection of labeled *K. pneumoniae* polysaccharide - complex(1-3). $C^{14}$ - labeled polysaccharide recovered by extraction of liver, spleen and adrenal partially retains the haptenic properties of the original polysaccharide(3). Apparently the bacterial polysaccharide(3), like $C^{14}$-labeled dextran in dogs (4), gradually disappears from animal tissues. Stark(5), however, studying mice injected with $C^{14}$-labeled pneumococcal polysaccharide, found the $C^{14}$ content of splenic extracts almost unaltered for one year, while the antigenic property of such extracts was markedly reduced. The present study of distribution of *K. pneumoniae* polysaccharide in 4 common laboratory animals is pertinent to the species variation in physiologic responses and antibody formation following injection of bacterial products.

*Materials and methods.* The $C^{14}$-labeled polysaccharide-complex was extracted with 0.01 N KOH from *K. pneumoniae*, type B, which had grown at 37°C for 16 hours upon agar media containing 1-$C^{14}$ acetate(3). The polysaccharide-complex had an isotopic activity of 0.678 $\mu$c/mg, was non-antigenic, but haptenic, and had no lethal toxicity. The polysaccharide was prepared as a 1% solution in 0.85% NaCl and sterilized by autoclaving. A single intravenous injection of the polysaccharide was given to guinea pigs (200 to 250 g body weight), Sprague-Dawley rats (100 to 130 g), Swiss albino mice (30 to 32 g) and New Zealand rabbits (2 to 2.5 kg). The rabbits received 0.5 mg/100 g body weight and the other species were given 1 mg/100 g body weight. Animals were sacrificed at 1, 3, 7, 14 and 30 days. Additional groups of guinea pigs and rats were given a single injection of the same quantity of polysaccharide by subcutaneous, intraperitoneal and intravenous routes and killed at 1, 3, 6, 12 and 24 hours and at 7 days. Determination of the $C^{14}$ in plasma and tissue was carried out as previously described(3). $C^{14}$ content is given as percent of injected dose and per gram dry tissue for liver, spleen and adrenals. The pooled mesenteric lymph nodes and pooled aliquots of liver of rats given a single intravenous injection of polysaccharide and killed at 7 days were ground in a Potter homogenizer and separated into insoluble and soluble portions by centrifugation. The polysaccharide fractions of lymph nodes and of liver were then removed from the soluble aqueous portion by the same technic used for extraction of the polysaccharide from *K. pneumoniae* and from liver, spleen and adrenal of the guinea pig(3). Precipitin tests were performed with heat-inactivated rabbit anti-Klebsiella serum and quantities of lymph node and liver extracts with $C^{14}$ content equivalent to original bacterial polysaccharide/ml of antiserum in 1:1 dilution with 0.85% sodium chloride solution. $C^{14}$ content of both precipitate and supernatant was determined(3). Controls

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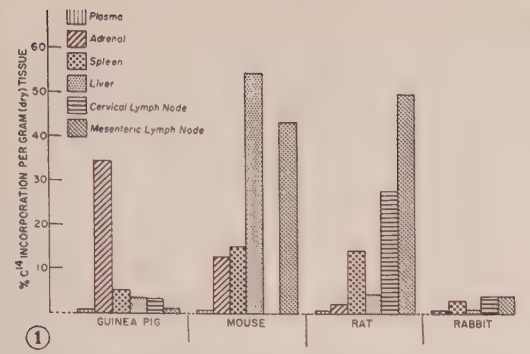
† Presented in part, at Am. Assn. Path. and Bact., Apr., 1957, Washington, D.C.



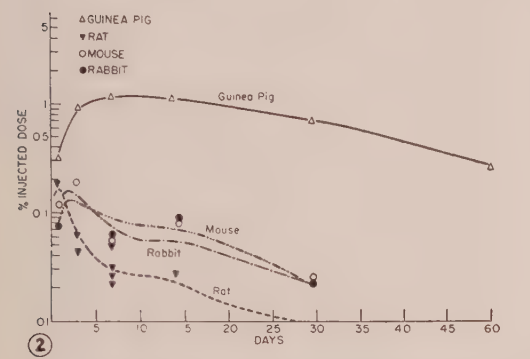




FIG. 1. % inj. $C^{14}$ in 1 g (dry wt) of tissue 7 days after single intrav. inj.

FIG. 2. Comparison of $C^{14}$ incorporation in adrenals of several animal species (intrav. inj.).

were a) comparable quantities of the original bacterial polysaccharide/ml of anti-Klebsiella rabbit serum and with normal rabbit serum diluted 1:1 with 0.85% sodium chloride and b) comparable quantities of tissue extracts with normal rabbit serum.

*Results.* Tissue distribution of $C^{14}$ from intravenously injected, labeled bacterial polysaccharide varied markedly in different species (Fig. 1). Maximum $C^{14}$ content/gram dry tissue was in the adrenal of guinea pig, liver and mesenteric lymph nodes of mouse, and mesenteric lymph nodes of rat (Fig. 1). $C^{14}$ uptake by spleen was relatively higher in mouse and rat than in guinea pig and rabbit. Isotopic content of tissues of rabbit was lower than those of the 3 other species. Of the 4 species, only the guinea pig showed marked adrenal uptake of $C^{14}$ (Fig. 1, 2). Negligible $C^{14}$ is present in thymus, salivary glands, thyroid, ovary, testes, myocardium, skeletal muscle, brain, pancreas or intestinal wall of these animals. In blood, the isotopic label was almost exclusively in plasma and not in leukocytes or erythrocytes.

After its intraperitoneal injection, the $C^{14}$-labeled polysaccharide entered the blood stream more rapidly in the rat than in the guinea pig (Fig. 3, 4). Plasma levels of $C^{14}$-labeled polysaccharide in the rat were comparable to those following intravenous injection. However, after its subcutaneous injection, the polysaccharide appeared more rapidly in plasma of guinea pig than of the rat (Fig. 3, 4). During the first 24 hours after intravenous, intraperitoneal or subcutaneous injection of $C^{14}$-labeled bacterial polysaccharide, incorporation of label into liver, spleen and adrenal of both guinea pig and rat tended to reflect the plasma levels. Regardless of route of injected labeled polysaccharide, the mesenteric lymph nodes of the rat showed a higher incorporation of $C^{14}$ than did the cervical nodes, while in the guinea pig the opposite was true.

The "polysaccharide" extracted from the mesenteric lymph nodes and liver of rats intravenously injected with labeled *K. pneumoniae* 7 days previously contained approximately 30% of the isotopic content of water soluble portion of tissue homogenate (Table I). The "polysaccharide" extracted from tissues had only a fraction of the specific activity of the original polysaccharide, but the labeled portion retained the haptenic quality (Table II).

*Discussion.* Variation in tissue distribution of different lipopolysaccharide endotoxins of Gram-negative bacilli may occur in the same as well as in different mammalian species. A considerable quantity of intravenously injected $P^{32}$-labeled *E. coli* endotoxin is taken up by liver and spleen of mice and guinea pigs(6). $I^{131}$-labeled somatic antigen of *Shigella flexneri* is also found in relatively high concentration in liver and spleen of the rat(7). However, Barnes, *et al.*(7), found little of the $I^{131}$ somatic antigen in the cervical lymph node of the rat. Braude, *et al.*(8), reported a greater uptake of $Cr^{51}$-labeled *E. coli* endotoxin into liver of rabbit than observed in the present study after injection of $C^{14}$-*K. pneumoniae* polysaccharide. The marked

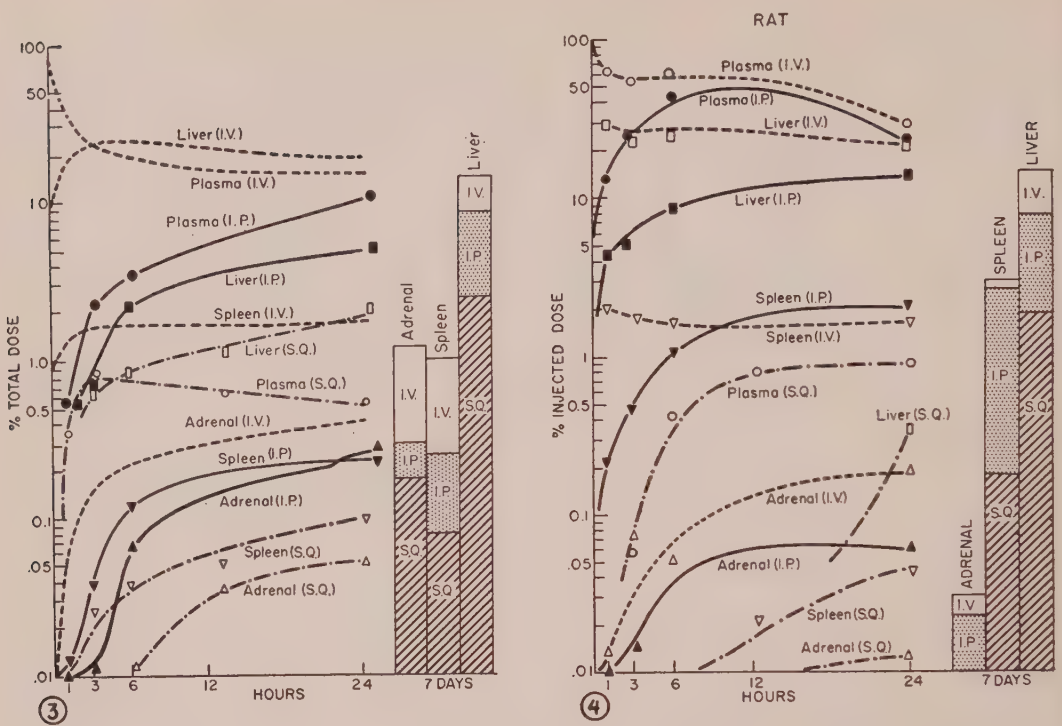



FIG. 3. Percent of $C^{14}$ from labeled *K. pneumoniae* polysaccharide in plasma, adrenal, spleen and liver of the guinea pig after intrav., intraper. and subcut. inj.

FIG. 4. Percent of $C^{14}$ from labeled *K. pneumoniae* polysaccharide in plasma, adrenal, spleen and liver of the rat after intrav., intraper. and subcut. inj.

uptake of label by the adrenal cortex after injection of $C^{14}$-labeled bacterial polysaccharide is found in the guinea pig, but not in rat, mouse or rabbit. In the guinea pig this adrenal uptake of $C^{14}$ is reduced to the level of the other 3 species by pre-treatment with cortisol, but not by certain other steroids or ACTH(2).

It is of interest that $C^{14}$-labeled dextran has a distribution in dogs similar to that of *K. pneumoniae* polysaccharide in rodents. Terry, *et al.*(4), have found relatively high concentrations of $C^{14}$-labeled dextran in lymph nodes, liver, adrenal and spleen of dogs. Apparently the dextran is metabolized in dogs, for there is a decrease of labeled polysaccharide in tissues of the dog and a continued respiratory loss of $C^{14}O_2$. The metabolism of $C^{14}$-labeled dextran has also been studied in mice by Gray(9). Whole body homogenates of mice show an incorporation of $C^{14}$ into protein and lipid, indicating the label had entered the general carbon pool.

Previous studies with $C^{14}$-labeled *K. pneumoniae* polysaccharide suggest that this bacterial product is metabolized by the guinea

TABLE I. Extraction of $C^{14}$-Labeled Polysaccharide from Mesenteric Lymph Node and Liver of Rat Injected with *K. pneumoniae* Polysaccharide and Days Previously.

| | | | | Tissue polysaccharide | | |
|---|---|---|---|---|---|---|
| | | Organ homogenate | Water-soluble fraction | Ethanol-insoluble, mg | $C^{14}$ recovered, % | $C^{14}$/mg, % of bact. poly. |
| Lymph node | mg | 328 | 137 | 16.9 | | |
| | mμc | 171 | 147 | 45.0 | 26.3 | .40 |
| Liver | mg | 3,280 | 1,900 | 50.4 | | |
| | mμc | 100 | 64.8 | 33.0 | 33.0 | .09 |

TABLE II. Quantitative Isotopic Precipitin Test with $C^{14}$-Labeled Polysaccharide Extracts from Mesenteric Lymph Node and Liver of the Rat.

| | Original bact. poly. | | Lymph node | | Liver | |
|---|---|---|---|---|---|---|
| Rabbit serum | Quantity, μg/ml AB | $C^{14}$ in precipitate, % | Quantity, μg/ml AB | $C^{14}$ in precipitate, % | Quantity, μg/ml AB | $C^{14}$ in precipitate, % |
| Normal | 20.0 | 2.5 | 6.0 | 1.0 | .14 | 1.9 |
| | 10.0 | 1.8 | | | | |
| | 3.0 | 1.8 | | | | |
| Anti-Klebsiella | 20.0 | 82.0 | 6.0 | 94.7 | .14 | 78.6 |
| | 10.0 | 91.0 | 3.0 | 98.0 | | |
| | 3.0 | 94.5 | | | | |
| | .1 | 90.0 | | | | |

pig(3). $C^{14}O_2$ is lost in the respired air during the first 48 hours after intravenous injection of the polysaccharide, while urinary loss of $C^{14}$ continues for as long as the tissues contain $C^{14}$. During the first week after injection, the $C^{14}$ in plasma declines exponentially to very low levels and following this, remains almost free of label. This observation, plus the failure of adipose tissue to incorporate $C^{14}$, suggests that relatively little of the $C^{14}$ from the labeled polysaccharide enters the general carbon pool during the metabolism of the *K. pneumoniae* polysaccharide. After injection of labeled *K. pneumoniae* polysaccharide, a considerable amount of $C^{14}$ in tissues of guinea pig and rat retains the original haptenic property. By simple qualitative extraction of liver and spleen of the guinea pig(3) and lymph nodes and liver of the rat, labeled polysaccharide is recovered which possesses part or all of the original haptenicity. Additional qualitative evidence for persistence of bacterial polysaccharide in animal tissues has been provided by the fluorescent antibody procedure for *K. pneumoniae* capsular polysaccharide(10) and pneumococcal polysaccharide(11) and by tissue extraction and precipitin titers with non-labeled pneumococcal polysaccharides in the mouse(12).

*Summary.* Tissue distribution of $C^{14}$-labeled *K. pneumoniae* polysaccharide-complex has been studied in guinea pig and rat after intravenous, intraperitoneal and subcutaneous injection, and in the mouse and rabbit after intravenous injection. The polysaccharide entered the blood stream from the peritoneal cavity more rapidly in the rat than in the guinea pig, while absorption from subcutaneous tissues was more rapid in the guinea pig. After intravenous injection, there were marked differences in tissue concentration of labeled polysaccharide in the 4 animal species. By simple extraction procedures, approximately 30% of the label in liver and mesenteric lymph nodes of the rat has been recovered in a polysaccharide retaining the haptenic property of the original polysaccharide.

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# Platelet Counts in the Rat After Hypophysectomy, Gonadectomy or Thyroidectomy.* (24168)

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It has been proposed that in mammals the number of circulating platelets, as well as erythrocytes and leukocytes, are under hormonal regulation(1-10). Bilateral removal of adrenal glands has been reported to cause an increase in platelet count(6-8); this increase has, however, been interpreted as due to surgical trauma(3). In human subjects administration of adrenal cortical extracts resulted in decreased platelet numbers(11), though no such effect was recorded following administration of adrenocorticotrophic hormone to either human beings or rats(7). Thus the role of adrenal hormones in control of circulating platelets has not been established. Reports are likewise conflicting on effects of gonadal steroids. Castration of rats resulted in decreased numbers of platelets, and administration of gonadal extracts in increased numbers(4). However, administration of large amounts of estrogens to either dogs or monkeys resulted in hypoplastic bone marrow and in a decrease in platelets(12). In human subjects injection of estrin and progesterone was reported to have no effect on the numbers of platelets(11), though more recently a decrease in platelets has been described after injection of estrogens, and an increase after injection of progesterone(10). Thyroxine has been reported to increase the number of platelets in human subjects(11); thyrotropic hormone also increased the number of blood platelets, but the increase appeared later and persisted longer(11). A significant decrease in platelet numbers has been described following removal of the pituitary(3).

In this study the role of pituitary, thyroid, and gonads has been reexamined in rats deprived of these organs. At the same time clot retraction was determined, in an effort to correlate numbers of platelets with this process.

*Methods.* Platelet counts were made weekly on male rats (Long-Evans strain) from the age of 5 weeks to 5 months. Ablation of endocrine organs was performed at 7 weeks of age.† *Hypophysectomy* was performed through the parapharyngeal approach and completeness of hypophysectomy was determined at autopsy by examination of sella turcica and by histological examination of target organs. No data from incompletely hypophysectomized rats is reported. *Castration* and *thyroidectomy* were performed surgically, the parathyroids being removed simultaneously with the thyroids. An intraperitoneal injection of 1 $\mu$c $I^{131}$ was given 20 hours before autopsy. All tissue from the ventral cervical region was removed and radioactivity was determined in a scintillation counter (13). The radioactivity of a comparable piece of thigh muscle was subtracted from that of the thyroid region and the difference represented iodine uptake by any thyroid remnant. Counts of less than 0.01% of the injected radioactivity (or 0.1% of the normal uptake) were regarded as evidence of completeness of thyroidectomy. *Platelet* numbers were determined by the Rees-Ecker method (14). Blood was obtained by direct puncture of tail vein with a hypodermic needle, after coating the site with vaseline to prevent platelet injury. Blood was drawn from the freely

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† All thyroidectomized, gonadectomized, and normal rats were maintained on a modified McCollum Diet I consisting of ground whole wheat 67.5%, casein 15%, skim milk powder 7.5%, sodium chloride iodized 0.75%, calcium carbonate 1.5%, melted fat 6.75%, fish oil (Vit. A and D concentrate) 1%. KI was added to 1 $\mu$g of iodine/g of diet (450 mg KI/liter, 300 ml/270.5 lb of diet). Hypophysectomized rats were maintained on Diet XIV consisting of ground whole wheat 68.5%, casein 5%, alfalfa leaf meal 10%, fish oil 5%, NaCl 1.5%. KI was added to 1 $\mu$g of iodine/g of diet (450 mg KI/liter, 300 ml/270.5 lb of diet). In addition a wet mash of modified McCollum Diet I was fed daily. All animals received a supplement of lettuce 2-3 times a week, and were maintained at 74° ± 1°F.

TABLE I. Platelet Counts after Gonadectomy, Thyroidectomy or Hypophysectomy. Thousands/mm³; 6 rats/group.

| | Weeks | Normal | Gonadectomized | Thyroidectomized | Hypophysectomized |
|---|---|---|---|---|---|
| Pre-oper. | 1 | 538 ± 18.6* | 558 ± 47.6 | 519 ± 32.7 | 509 ± 16.5 |
| Post-oper. | 1 | 597 ± 35.6 | 601 ± 23.6 | 477 ± 28.0 | 379 ± 2.0† |
| | 2 | 499 ± 9.4 | 436 ±106.8 | 487 ± 32.4 | 383 ± 19.8† |
| | 3 | 536 ± 42.2 | 381 ± 55.6 | 352 ± 74.0† | 354 ± 52.8† |
| | 4 | 490 ± 44.3 | 532 ± 45.6 | | 381 ± 37.2† |
| | 6 | 522 ± 26.0 | 425 ± 4.8 | | 344 ± 51.2† |
| | 12 | 552 ± 21.7 | 517 ± 13.9 | 375 ± 18.4† | 371 ± 43.5† |

* S.E. $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$. † $P < .01$.

bleeding wound into a Trenner automatic white blood cell diluting pipette; the blood was diluted immediately and shaken gently for 2 minutes. The first drops from the pipette were discarded; 15 minutes were allowed for platelets to settle in haemocytometer before counts were made. Duplicate determinations made from the same animal did not differ by more than 4%. *Clot retraction* was determined at the end of the experimental period by the method of Budtz-Olsen(15). Under ether anesthesia 5 cc of blood were drawn from the aorta into a paraffin coated syringe. A known quantity of blood was then allowed to run into a fluid of approximately the same specific gravity as of whole blood (mixture of liquid paraffin and trichloroethylene). The fluid containing the suspended sphere of blood was maintained at 37°C during clot retraction. The extruded serum of lower specific gravity rose to a higher level as the clot sank. When clot retraction was complete the expressed serum was removed by pipette and measured in a graduated cylinder. As a check the clot was lifted, washed in ether, and its volume determined by water displacement. Clot retraction was expressed as percent of serum extruded from whole blood.

*Results.* The effects of castration, thyroidectomy and hypophysectomy on numbers of blood platelets are shown in Table I. Only thyroidectomy and hypophysectomy resulted in significant decrease; platelet counts were reduced and eventually reached the same low level after 12 weeks. The decrease in platelet count occurred almost immediately after hypophysectomy, whereas after thyroidectomy it occurred only after the third week. No significant difference was observed in clot retraction in gonadectomized animals, but it was impaired in both thyroidectomized and hypophysectomized rats (Table II). The impairment was greater in thyroidectomized rats suggesting that the residual activity of thyroid after hypophysectomy was able to influence clot retraction. The change in clot retraction is consistent with the assumption that a reduction in platelets impairs normal retraction of the blood clot(5).

TABLE II. Clot Retraction after Gonadectomy, Thyroidectomy or Hypophysectomy.

| Group | No. of rats | Clot retraction, % serum |
|---|---|---|
| Normal control | 6 | 42 ± 3.0 |
| Gonadectomized | 6 | 44 ± .8 |
| Thyroidectomized | 6 | 22 ± 1.5 |
| Hypophysectomized | 3 | 33 ± 3.4 |

The number of circulating platelets is therefore controlled, at least in part, by pituitary and thyroid secretions. As the number of circulating platelets is decreased after thyroidectomy as well as after hypophysectomy it is probable that the reduction in platelets after hypophysectomy is secondary to thyroid deficiency, however it can not be ignored that alterations in pituitary function occur after thyroidectomy, and reduction in numbers of platelets may result from this alteration.

It should be noted that besides platelets the only formed elements in blood which decrease in numbers after hypophysectomy are erythrocytes.

*Summary.* Removal of pituitary gland, or of thyroid resulted in decrease in number of

circulating blood platelets. Decrease in clot retraction observed in these animals was consistent with reduced platelet counts. Gonadectomy had no effect on post-operative platelet counts or clot retraction.

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## Influence of X Irradiation on Potassium Retentivity by *Escherichia coli.** (24169)

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Hevesy and Zerahn (quoted by Hevesy (1)) observed that the $K^+$ efflux from yeast increased after X irradiation. Bruce and Stannard(2,3) made a detailed study of the influence of radiation on $K^+$ efflux and influx in yeast. The present study is an extension of their work to bacteria. Although a number of different species were tested, this report will be concerned only with *Escherichia coli.* Strain B and its radiation-resistant mutant B/r are compared, and some other factors that influence $K^+$ retentivity by *E. coli* are discussed.

*Methods. Escherichia coli* was cultivated in broth of the following percentage composition: glucose, 1; $(NH_4)_2HPO_4$, 0.4; $KH_2PO_4$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.07; and sodium citrate, 0.05. Glucose and ammonium phosphate were autoclaved separately as 20 and 8% solutions, respectively, then added to the autoclaved and cooled basal solution. A 12-hour, 500-ml culture was decanted into 5 liters of fresh broth contained within a 12-liter round-bottom flask in 37°C water bath. The resulting mixture was continuously aerated through a 4-mm bore glass tube at 2½ liters of air per minute. After 12-14 hours incubation, the cells were harvested in a Sharples centrifuge, then mixed with enough distilled water to form about 40 ml of thick suspension. This will be designated as stock suspension. Cells were washed in centrifuge once with distilled water, twice with 0.05 *M* sodium phosphate (pH 6.2) and, after the last centrifugation, were made up to 200 ml in this buffer. The suspension was divided in half and each 100-ml portion was placed

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in a Petroff flask of this capacity. One flask was affixed to a General Electric Maxitron X-ray apparatus as described by Bruce(3); the other flask served as control. Both were oxygenated through a 2-mm bore glass tube at 1 liter of oxygen per minute. This served as a means of agitation as well as maintaining high oxygen tension. Irradiation of test suspension was begun after 15 minutes of oxygenation, and oxygenation of both suspensions was continued during the irradiation period. The G. E. unit was operated at 250 kvp and 30 ma with 1 mm of added aluminum between target and test suspension; the unit delivered X-rays at 4000 r per minute. Temperature of test and control suspensions was maintained at 22°-25°C by water baths. After various doses of radiation, 10-ml aliquots of test suspension were transferred from Petroff flask to 15-ml centrifuge tubes. These aliquots were centrifuged at 4000 rpm for 10 minutes and the clear supernatants carefully decanted into test tubes. The cell pellets were immediately resuspended to volume (10 ml) with 0.02 *M* ammonium phosphate (pH 5.2); they were again centrifuged, and supernatants collected as before. In this manner, potassium ions were extracted from the bacteria. Each cycle of events required 20 minutes—10 minutes for centrifugation and decanting, and 10 minutes for resuspension and holding prior to subsequent centrifugation. The 20-minute centrifugation cycles were continued for a minimum of 3 hours; thus at least 10 supernatants were collected from each aliquot. An aliquot from control suspension was manipulated in like manner. The viable-cell count did not change appreciably as a result of these manipulations. Operations were carried out at room temperature, about 22°C. The series of supernatants in test tubes were analyzed for $K^+$ content by a flame photometer (Baird Associates) with suitable correction for $Na^+$ in the first supernatant and for $NH_4^+$ in subsequent supernatants. Ammonium ions, however, affected photometer readings very little. Summation of $K^+$ content of successive supernatants permitted construction of efflux-time curves. In these curves, amount of $K^+$ effluxed (mmoles) per given wet weight (kg) of cells as plotted *vs.* time (hours). Cell concentration will be expressed as mg (wet weight) of cells/ml of suspension. Cell concentration of stock suspension was determined by hematocrit reading on a diluted ½-ml aliquot, with suitable corrections for dilution. (Specific gravity of cells was assumed to be unity.) Cell concentration of this stock was always about 200 mg/ml, equivalent to a viable count of $1.5 \times 10^{11}$ cells/ml. To avoid introducing cell concentration as a variable during efflux measurement and to ensure concentration of $K^+$ sufficiently high for flame photometry, the cell concentration during efflux was always set to 40 mg/ml. When, as outlined above, the entire stock was used, the cell concentration during irradiation was 40 mg/ml and aliquots from Petroff flasks were used directly for efflux measurement. However, when lower cell concentrations were used during irradiation it was, of course, necessary to concentrate the cells prior to efflux.

*Results.* We discovered that little or no potassium efflux occurs in the presence of $Na^+$ but efflux will occur readily in the presence of $NH_4^+$. Thus we used sodium phosphate as buffer during irradiation to minimize efflux during this interval and substituted ammonium phosphate thereafter (when efflux was to be measured). The first points in Fig. 1 (0 hours) represent quantity of $K^+$ in 0.05 *M* sodium phosphate (first supernatants). Only a very small amount of potassium has effluxed, whereas the efflux begins immediately upon replacement with ammonium phosphate. During the first hour the slope fluctuates, but after 1½ hours, the curves become linear and a numerical expression for efflux rates may be obtained.

The control efflux rate of *E. coli* B obtained in different experiments varied from 0.17 to 0.28 mmole of $K^+$/kg of cells/hour, but since the efflux rates after a given dose of radiation varied by a corresponding amount, data from different experiments could be compared, if the results were expressed as percentage of normal $K^+$ retentivity. (This expression is obtained by dividing control efflux

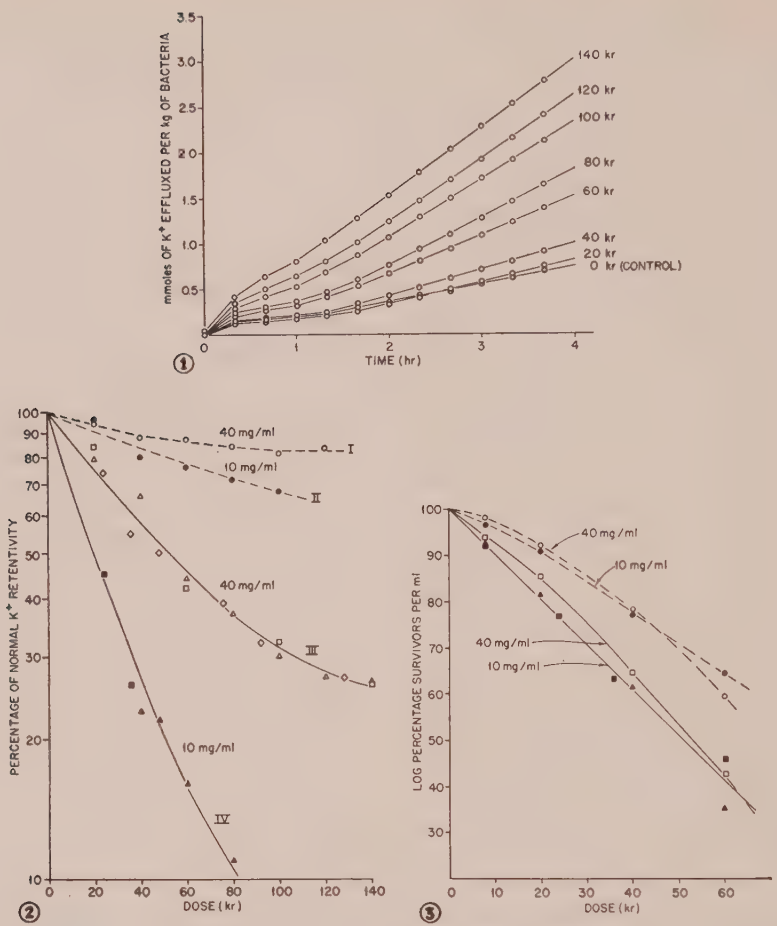


FIG. 1. Effect of X-irradiation on potassium efflux from *Escherichia coli* B. Cell concentration during irradiation was 40 mg/ml. For the 140-kr curve, efflux rate was 0.74 mmole/kg/hr; 120-kr curve, 0.71 mmole/kg/hr; 100-kr curve, 0.63 mmole/kg/hr; 80-kr curve, 0.52 mmole/kg/hr; 60-kr curve, 0.43 mmole/kg/hr; 40-kr curve, 0.29 mmole/kg/hr; 0-kr curve (control), 0.19 mmole/kg/hr.

FIG. 2. Effect of X-irradiation on potassium retentivity by *Escherichia coli* B and B/r at different cell concentrations during irradiation. Solid line, *E. coli* B; broken line, *E. coli* B/r.

FIG. 3. Effect of X-irradiation on survival of *Escherichia coli* B and B/r at different cell concentrations. 40 mg/ml equivalent to initial viable cell count of $3 \times 10^{10}$/ml; 10 mg/ml equivalent to initial viable cell count of $7 \times 10^{9}$/ml. Solid line, *E. coli* B; broken line, *E. coli* B/r.

rate by the efflux rate at a given dose and multiplying by 100; Bruce and Stannard (2).) The data from Fig. 1 have been expressed in this manner and have been plotted semilogarithmically in Fig. 2 (curve III, triangles); squares and diamonds represent corresponding data from 2 other experiments. Good reproducibility was obtained in experiments in which cell concentration was set at 40 mg/ml. Control efflux rate at 10 mg/ml varied from 0.04 to 0.09 mmole/kg/hour; this low rate of efflux was difficult to measure with the flame photometer and rendered the corresponding values for percentage normal retentivity less accurate. (All cell concentrations were those used during irradiation; as has been pointed out, cell concentration during efflux was always 40 mg/ml.) *E. coli* B/r had relative high control efflux rates; at 40 mg/ml about 0.60 mmole/kg/hour, and at 10 mg/ml 0.67 to 0.98 mmole/kg/hour. Good reproducibility in potassium retention

calculations was obtained at both cell concentrations. Since X irradiation of B/r increased efflux rate very little, the decrease in percentage normal retentivity with increasing dose was less pronounced than with strain B (compare curves I and II with curves III and IV, Fig. 2). It may also be noted in Fig. 2 that magnitude of radiation damage varies inversely to cell concentration in the irradiated suspension and, lastly, that the slope of retentivity curves decreases at higher doses.

The increase in $K^+$ efflux brought about by irradiation is not caused by gross cell lysis, because (1) diluted aliquots from the irradiated suspensions were no less turbid than similarly diluted aliquots from control suspensions, and (2) supernatants from irradiated suspensions contained no more 2600 A absorbing material than supernatants from control suspensions.

The influence of radiation and cell concentration on viability is presented in Fig. 3. (Use of same symbols on a curve in Fig. 3 as in Fig. 2 indicates that the data are from a common experiment.) The influence of cell concentration on $K^+$ efflux noted in Fig. 2 apparently has no equivalent effect on viability, although at lower cell concentration survival curves are more nearly exponential. Experiments by Hollaender *et al.*(4) demonstrated that the shape of such curves can be influenced by oxygen tension. Thus the downward concavity of curves at high cell concentration may be caused by oxygenation's insufficiency to meet the oxygen demand of this large number of cells.

The present study does not permit conclusions as to site of potassium retention damage. Possibly, damage is to the cell membrane, as Bruce(3) considered to be the case with yeast; or the damage may be to an intracellular metal-organic complex responsible for binding potassium, denaturation inducing an irreversible loss of the metal ion. Indeed, the researches with bacteria by Cowie (summarized by Roberts *et al.*(5)) and Eddy and Hinshelwood(6) make the latter idea especially attractive.

*Summary.* 1) Potassium efflux from stationary-phase cells of *Escherichia coli* takes place readily in the presence of ammonium ions. Hence, through the use of ammonium phosphate buffer, $K^+$ efflux was studied at a set value of pH. Sodium ions, on the other hand, retard $K^+$ efflux, a finding that has been used to advantage when sodium phosphate is used as buffer in delaying efflux measurement. 2) Potassium efflux from *E. coli* increases as a result of X irradiation. Amount of X-ray damage to $K^+$ retention mechanism varies inversely with cell concentration during irradiation. *E. coli* strain B is much more susceptible to this radiation damage than is its mutant B/r.

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# Effect of Tannic Acid and Adsorbed Purified Blood Group Substances on Human Erythrocytes.* (24170)

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During recent years, many immunological tests have been described in which use was made of tannic acid-treated erythrocytes to adsorb various antigens from solution. It was our purpose to study the effect of tannic acid on the red cell itself by observing changes in serological reactivity of the A, B, C, D, E, M and N antigens of human erythrocytes following the tanning process. In addition, tanned human red cells were studied for their ability to adsorb purified blood group A and B substances; this portion of the work is an extension of the findings of Brading(1) who found that blood group A and B substances could be adsorbed onto group O cells.

*Materials.* Several diluents were employed: 0.85% NaCl unbuffered or mixed with equal volume of $\frac{M}{15}$ $Na_2HPO_4 \cdot KH_2PO_4$ solutions to produce pH 7.2 or 6.4; and serum-saline prepared by mixing with 0.85% saline serum from a person having type AB blood to final serum concentration of 0.4 or 1%. The human A substance (cyst 9 phenol-soluble fraction) was isolated from a human ovarian cyst fluid(2) by phenol extraction method of Annison and Morgan(3); the human B substance, also of cyst origin, was obtained through the kindness of Dr. Sidney Leskowitz of Mass. General Hospital. Each week a 1:1000 stock solution of Merck USP fluffy tannic acid was prepared and daily working solutions were made by dilution of this stock, using 0.85% NaCl. Human red cells of types O, A, and B were collected directly into acid citrate dextrose preservative (ACD) solution, stored at 0°-4°C and used until they were 3 weeks old, since it was determined that cells over 4 weeks old were too fragile for use. Cells to be tested for factors C, D, E, M, and N were used within 2 hours after collection. All antisera were commercially prepared, "saline agglutinating" typing sera and all were of human origin except anti-M and anti-N which were rabbit antisera.

*Method.* Red cells were washed 3 times in saline and a 2.5% suspension prepared in buffered (pH 7.2) saline. An equal quantity of appropriately diluted tannic acid solution was added to this suspension, which was then shaken and incubated in water bath at 37°C for 15 minutes. The cells were packed by centrifugation for 2 minutes at about 1300 rpm and washed once in buffered (pH 7.2) saline. A solution of the blood group substance in buffered saline (pH 6.4) was added to the packed tanned cells in volume sufficient to give a 4% cell suspension. After 15 minutes at 37°C for adsorption the cells were washed once in 0.4% human AB serum as recommended by Brading(1) to prevent hemolysis and aid in resuspension. For agglutination, a 2.5% cell suspension was made in 0.4% AB serum and to 0.2 ml of this was added 0.2 ml of antiserum diluted in 1% AB serum; where required, controls of untreated cells and cells treated with tannic acid alone were used. The tubes were shaken, incubated 30 minutes at 37°C, reshaken and centrifuged for 2 minutes at about 1300 rpm, then examined macroscopically and at magnification of 30x. The results were recorded as follows: 4+, a single heavy clump of packed cells that did not disperse on gentle shaking; 3+, several gross clumps; 2+, clumps small but clearly visible macroscopically; 1+, clumps well defined at 30x, all cells clumped; ±, scattered clumps at 30x; —, no clumps at 30x, cells evenly dispersed on gentle shaking. In titrating with anti-D serum, the saline tube procedure was followed as outlined by American Asso. of Blood Banks(4); the results were recorded as for anti-A and anti-B titrations. In the case of C and E antigens, 2 drops of a 2.5%

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cell suspension were added to 1 drop of antiserum, shaken, incubated at 37°C for one hour and centrifuged 1 min. at about 1000 rpm; the results were reported as positive or negative after examination with 10x hand lens. M and N antigens were tested in similar mixtures after incubating 30 minutes at room temperature, and examined immediately for clumps without centrifugation; the results were recorded as positive or negative.

*Results.* To test the effect of tanning on surface antigens, human red cells possessing antigens A, B, C, D, E, M, and N were treated with a 1:20,000 dilution of tannic acid. The cells were then tested for ability to agglutinate with homologous antiserum. When tested against anti-A, anti-B, and anti-D, the tanned cells agglutinated as well as untanned cells and the titers of antisera were the same in each case. In contrast, the anti-E, anti-M, and anti-N sera failed to agglutinate tanned cells; since these commercial antisera were relatively weak, it was not practical to carry out a titration and the possibility, therefore, cannot be ruled out that a sufficiently potent antiserum might have given some agglutination. Brading(1) found that tanning the cells reduced anti-A, anti-B and anti-D titers; we have no explanation for the difference between her results and ours.

Before extensively studying adsorption of purified blood group substances onto tanned cells, a single system (A substance adsorbed onto O cells) was studied to determine the most suitable concentration of tannic acid and blood group substance. Cells treated with tannic acid in concentrations varying from 1:2000 to 1:40,000 and then incubated with 1% solution of A substance all gave the same (1:32) titer with anti-A serum. However, when the A substance was added, there was some hemolysis in those cells treated with tannic acid concentrations of 1:10,000 or higher. Since the use of tannic acid concentrations greater than 1:20,000 did not increase the reactivity of the cells toward anti-A serum, this concentration was selected for sensitization in subsequent work.

Group O cells treated with tannic acid 1:20,000 were then incubated with blood group A substance in concentrations varying from 0.01% to 2%. It was found that the anti-A titer was constant when the blood group concentration used for adsorption varied from 0.1% to 2%, but was distinctly reduced at the 0.01% level. It should be noted that untanned O cells did not adsorb blood group A substance. From this experiment a concentration of 1% blood group substance solutions was considered to be optimal.

TABLE I. Effect of Adsorption of Blood Group Substances on Erythrocytes of Groups A and B.

| Cells | Titer *vs* anti A | Titer *vs* anti B |
|---|---|---|
| Untreated A cells | 1/64 | |
| A cells + 1% B sub. | " | |
| Tanned A cells | " | |
| *Idem* + 1% B sub. | 1/16 | 1/32 |
| Untreated B cells | | 1/256 |
| B cells + 1% A sub. | | " |
| Tanned B cells | | " |
| *Idem* + 1% A sub. | 1/16 | 1/32 |

Note: Titer represents highest dilutions giving a 1+ reaction.

Tanned group A cells were then treated with B substance, tanned group B cells with A substance, and both groups of cells were then tested against anti-A and anti-B sera; the results are recorded in Table I. The adsorption of blood group substance onto tanned cells resulted in a lowered antiserum titer against the antigen originally contained on the red cell and the added ability to agglutinate with the antiserum to the adsorbed antigen. There was no change in serum titer to the original or adsorbed antigens when concentration of blood group substance was increased from 1 to 2%. The group A and group B cells, therefore, simulated group AB cells in their reactivity.

Since in both cases studied there was a partial suppression of the reactivity of the original erythrocyte antigen, the question was raised whether or not this was specific or some general effect on reactivity of the red cell. B substance was adsorbed onto A cells possessing the D antigen, and from Table II it can be seen that reactivity of the D antigen was unaffected.

*Discussion.* Since many antigens are known to be present on, or close to, the surface of the red cell, they must obviously oc-

TABLE II. Effect on Anti-D Titer of Adsorption of Blood Group B Substance.

| A cells | Titer of anti-D serum | | | | | | |
|---|---|---|---|---|---|---|---|
| | U | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |
| (D) + cells in saline | 3 | 4 | 4 | 3 | 2 | 2 | — |
| (D) + cells in tannic acid | 3 | 4 | 3 | 3 | 2 | 2 | — |
| (D) + cells, tanned, + 1% B | 4 | 4 | 3 | 3 | 2 | 2 | — |
| (D) + cells, + 1% B | 4 | 4 | 4 | 3 | 2 | 1 | — |
| (d) − cells in saline | — | — | — | — | — | — | — |
| (d) − cells in tannic acid | — | — | — | — | — | — | — |
| (d) − cells, tanned + 1% B | — | — | — | — | — | — | — |
| (d) − cells + 1% B | — | — | — | — | — | — | — |

cupy limited and specific sites. Little is known concerning distribution of these antigens or whether they occupy one or more areas, although it may be presumed that if a cell can agglutinate that the antigen involved occupies at least 2 distinct sites. Tanning of the red cells sorted out the antigens tested into those that were unaffected by such procedure (A, B, and D antigens) and those whose activity as determined by reaction with homologous antisera was eliminated or markedly reduced (C, E, M, and N antigens). This would seem to indicate that the tannic acid was attached on or close to the latter 4 antigens; it further points up a difference in location of the 3 Rh antigens tested, the C and E antigens being quite distinct from the D antigen.

The A, B, and D antigens, while unaffected by tannic acid, react differently when the A and B substances are adsorbed onto the tanned cells. The A and B antigens already part of the cell surface are somewhat reduced in activity while the D antigen is unaffected. One possible explanation is that the adsorbed substances partially block the indigenous antigen but that the D antigen is sufficiently remote so that its activity is undisturbed.

It is interesting to note that adsorption of blood group substances onto red cells does not take place unless the cells are tanned. Since in most instances polysaccharides will adsorb to untanned cells, it would appear that the polypeptide portion of the blood group substance is probably important for the adsorption process while apparently unnecessary for the serological specificity. It is of further interest that adsorption of A or B substances onto cells of group B or A converts them to cells indistinguishable serologically from cells of group AB.

*Summary.* When human erythrocytes were treated with tannic acid, they reacted normally with anti-A, and anti-D sera, but the reactions with anti-C, anti-E, anti-M and anti-N were either eliminated or markedly reduced. Purified blood group A substance could be adsorbed onto tanned B cells and B substance onto tanned group A cells; in each case the cells reacted serologically as group AB cells. When the D antigen was present along with the A antigen, the former was unaffected by adsorption of B substance.

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# Electron Microscopy of Equine Abortion Virus. (24171)

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That strain of equine abortion virus (EAV) which has been adapted to the Syrian golden hamster provides, together with this animal, an excellent biological system for a variety of studies of virus-host relationships. One of the many advantages of the system is the extreme rapidity with which the virus acts to produce a fatal disease in hamsters, invariably culminating in death of animals in 18 to 21 hours after inoculation. Thus, the time factor involved during the disease precludes many undesirable side effects, such as anorexia. Systematic and reproducible studies, both cytological and biochemical, may readily be made of the sequential changes which occur. The pathological picture, both gross and cellular, is unmistakable. These changes have been described elsewhere(1,2), but it is of interest to note here that substantially all the hepatic cells of infected animals contain well-defined intranuclear inclusions. Certain phases of investigations employing EAV have been hampered by the fact that the morphology of the virus, as well as the number and intracellular orientation of the elementary bodies, has been unknown. It has been difficult to interpret the observed sequential biochemical changes in the host cells(3). The question has been whether these changes are due principally to the obligate biochemical response of the host cells to the presence of the virus, or to the simple physical presence of large numbers of virus particles. The present study was undertaken with several purposes in view: to elucidate the morphology of EAV; to gain some knowledge of the number and site of the elementary bodies parasitizing the host cells; and to attempt electron microscopic visualization of the characteristic intranuclear inclusion bodies seen by light microscopy.

*Materials and methods. Virus.* Material from the 186th passage of EAV in hamsters comprised the inoculum. The virus and inoculation of animals has been previously described(2). *Electron microscopy.* Small portions of liver removed from ether-anesthetized animals were cut into small blocks and fixed by immersion for 2 hours in chilled 1% osmium tetroxide buffered by veronal acetate approximately to pH 7.4. Following dehydration in graded alcohols, the blocks were embedded in a mixture of 25% methyl-75% butyl methacrylate catalyzed with benzoyl peroxide, sectioned on a Porter-Blum (Servall) microtome and examined with an RCA Model EMU-2B electron microscope.

*Results. The virus.* Structures which were presumably virus particles or stages in development thereof were seen in several forms. These forms may be categorized as: (1) poorly defined "nucleus," with no outer membrane, as shown in (a) of Fig. 1; (2) double membrane with apparently no "nucleus," as exemplified by (b) in Fig. 1; (3) well-defined "nucleus," surrounded by a single membrane, as seen in (c) of Fig. 1; (4) dense "nucleus" surrounded by a double membrane, shown in (d) of Fig. 1 and 2. It should be noted that some of these apparently different forms may be due, in actuality, to the plane of sectioning and/or the level of focus. The structures were ovoid, and those possessing a double membrane (or halo) extended approximately 146 mμ in diameter across the long axis to the limits of the outer membrane. The inner membranes measured approximately 90 mμ across the long axis, and the dense central bodies, or "nuclei," averaged about 40 mμ in diameter. Compression by the microtome knife probably was responsible for the ovoid shape of the particles. The basis for considering the observed structures

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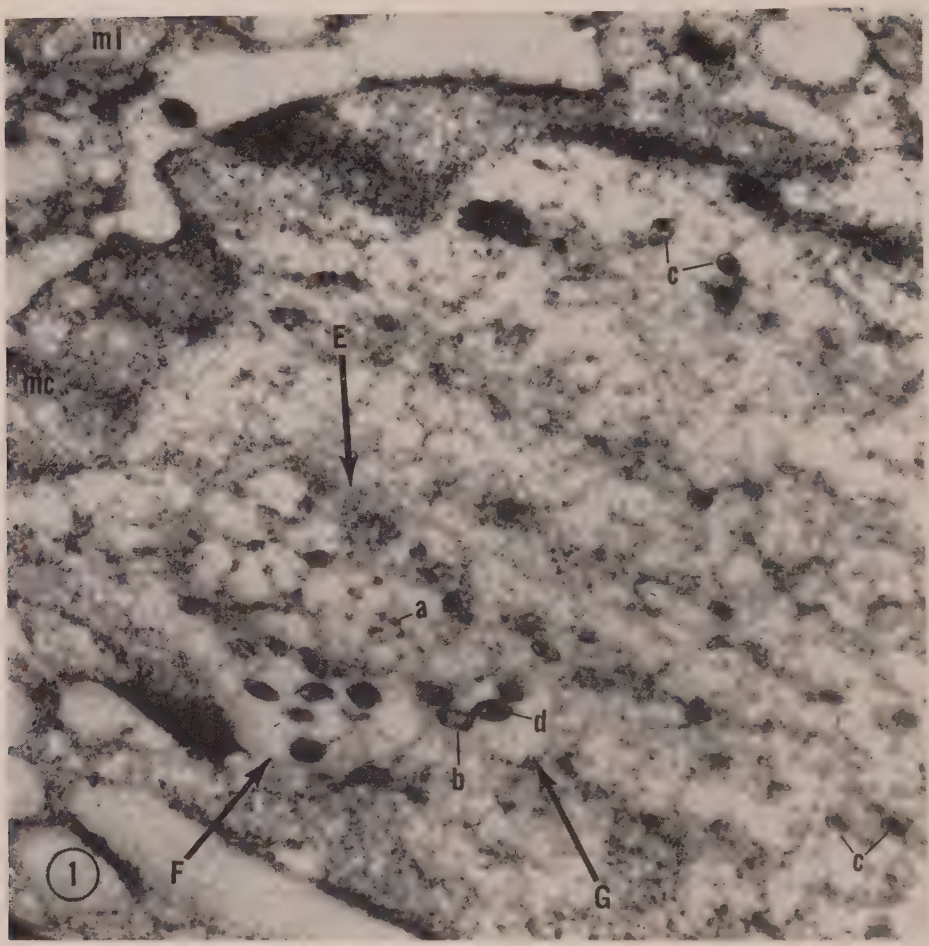


FIG. 1. Hepatic parenchymal cell of hamster inoculated with EAV. Various forms of intranuclear virus particles are shown, (a), (b), (c) and (d), as well as 3 viral "nests," (E), (F) and (G). Note margination of chromatin (mc). Mitochondrion (mi). Osmium fixation. 42,000 ×.

to be virus particles is predicated principally upon the fact that they are notably absent in control sections (Fig. 3) and are present in all examined sections of infected tissues.

*Orientation of virus.* A striking feature, readily apparent in Fig. 1, is that the elementary bodies are present in large numbers in the nuclei of the host cells, but were noticed only occasionally in the cytoplasm. Three distinct, although contiguous, "nests" may be discerned in the lower left quadrant of the nucleus in Fig. 1. One of these (e) seems to be composed of small central bodies enmeshed in a rather coarse reticulum; the other 2 ("f" and "g") contain structures which are considered to be more or less mature virus particles grouped together in vacuolated areas.

*Cellular pathology. Inclusion bodies.* No intranuclear inclusion bodies either of amorphous or crystalline structure were recognizable in our electron micrographs. *Margination of chromatin.* The represented figures of infected cells clearly show margination of chromatin around the periphery of the nuclei. The arrangement, thickness, and density of the marginated chromatin closely parallels that observed by light microscopy. *Nuclear membranes.* The nuclear membranes appeared distorted and were discontinuous in several areas. A comparison with a normal control (Fig. 3) serves to emphasize this cytopathogenic effect. *Cytoplasm.* Although

the infected cells apparently are undergoing general disorganization and degeneration, it is of interest to note that mitochondria, prominent in the control section (Fig. 3), appear less conspicuous in the cytoplasm of infected cells. Large, well-defined vacuoles are present in the cytoplasm of the infected cells.

*Discussion.* As more and more types of animal viruses are studied *in situ* with host cells by electron microscope a common structural pattern has become apparent. The virus particles are generally typified by a dense central body surrounded by one or two rings. Examples which may be cited include herpes

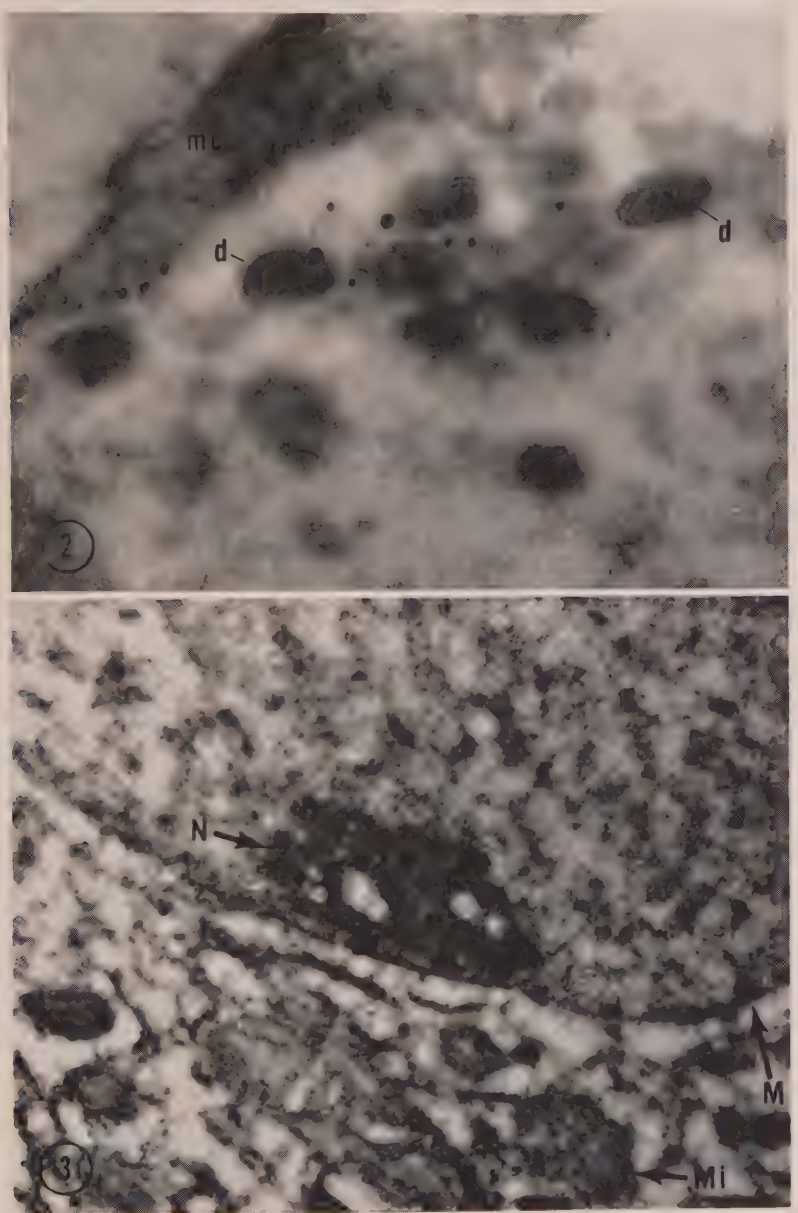


FIG. 2. Hepatic parenchymal cell of inoculated hamster showing double-ringed form of intranuclear elementary body, (d). Marginated chromatin (mc). Osmium fixation, 89,000 ×.

FIG. 3. Hepatic parenchymal cell of normal control hamster. Nuclear membrane (M), nucleolus (N), mitochondrion (Mi). Osmium fixation. 23,000 ×.

simplex reported by Morgan, *et al.*(4), influenza virus also observed by Morgan and co-workers(5), the salivary gland viruses reported by Luse and Smith(6), and the virus-like structures seen by deHarven and Friend in mouse leukemia material(7), and by Fawcett in frog adenocarcinoma(8). The EAV herein reported conforms very closely to the pattern. These examples of correlation in structure of different types of animal viruses serve to accent the importance of the questions raised by Morgan(9) concerning the relationship between structure and function.

The subject of intranuclear inclusion bodies associated with viral lesions has been of sustained interest to virologists for many years. Until recently no incontrovertible evidence for the existence of viral inclusion bodies in infected cells had been demonstrated by electron microscopy. It may be, at least in some instances, that the substances comprising the inclusion bodies which are readily seen by light microscope are too similar in electron density to the surrounding nuclear material to be visualized by the electron microscope. However, conclusive electron microscopic evidence of intranuclear inclusion bodies has been presented recently by Fawcett, *op. cit.*, and by Luse and Smith, *op. cit.* It is of considerable interest that margination of chromatin was prominent in the renal tumor cells studied by Fawcett, but was not a feature of the infected salivary gland cells employed by Luse and Smith. In contradistinction the EAV material, although revealing no intranuclear inclusion bodies did show conspicuous margination of chromatin. Whether these contrasting characteristics are features of the different viruses or of the different host cells constitutes a problem which might be explored.

*Summary.* 1) Young Syrian golden hamsters were inoculated with equine abortion virus (EAV). Hepatic cells from these animals, in late stages of infection, were studied with the electron microscope, and compared with similar material from uninfected (control) animals. 2) Various forms in which virus particles were seen, are described, and their similarity to other viruses is discussed. 3) Virus particles were observed predominantly in the nuclei. 4) "Nests" of virus particles were seen, but these were not considered to be equivalent to the intranuclear inclusion bodies characteristically seen by light microscopy. No structures identifiable as inclusion bodies were seen. 5) Other cytopathogenic effects are described.

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## Maintenance of Gonads of Frog Larvae on Synthetic Media.* (24172)

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Recent studies on gonads of adult and larval amphibians have shown that these organs can be grown and maintained *in vitro* on media composed of chick embryo extract and cock plasma, and on chick embryo extract and agar(1). To determine what factors influence differentiation of the amphibian gonad,

* Aided by grant from U.S.P.H.S.

synthetic media were employed as nutrients, eliminating possible effects of unidentified substances in media obtained from natural sources. The object of our study was to learn which of several chemically defined media could best maintain *in vitro* the sexually differentiated gonads of larval anurans.

*Materials and methods.* Ovaries and testes of second-year tadpoles of *Rana catesbeiana* were placed in watch glass cultures(2). Nutrient media used were T.C. 199(3), T.C. 1066 (communication from Connaught Medical Lab.) White's Nutrient Solution(4) an amino acid base medium containing purines and pyrimidines of the nucleic acids, which we developed and designated AAB, and Tyrode's solution. Larvae were made bacteriologically sterile by use of antibiotics and sulfa drugs before removal of gonads. Testes cultured were usually whole organs, while each ovary was cut into 3 parts before being placed in culture. Explants of gonads were placed on strips of cellulose acetate fabric and these laid on solid substrate containing the nutrients(5). A total of 56 testes and 320 ovarian explants were kept in cultures for periods varying 4 to 28 days. Numbers of explants on various media and lengths of culture periods are given in Table I. Culture media were prepared by putting 9 parts nutrient solution and 1 part penicillin solution (200-300 $\mu$g) in the watch glass, adding 10 parts 1% agar solution with phenol red indicator, stirring and allowing the mixture to solidify. Cultures were kept at 21°C and pH 7.2. Organs were transferred to fresh media each fifth day.

*Results.* Criteria for determining effectiveness of each medium were degree of maintenance of large ovocytes (auxocytes) and young germ cells, and maintenance and proliferation of non-germinal cells. Chick embryo extract and cock plasma proved more satisfactory for growth of ovaries and testes than the other media used. Characteristic changes in cellular pattern were associated with each medium and outgrowths of fibroblasts from explants were noted in all.

On T.C. 199 most auxocytes degenerated during the first 2 weeks in culture and few young germ cells were observed, but there was an increase in number of non-germinal cells which filled in the areas vacated by the germ cells. Immature ovarian germ cells appeared essentially normal when grown on T.C. 1066, even after 28 days in culture; but auxocytes underwent cytolysis. The same increase in number and size of follicular cells occurred on both of these media. White's nutrient solution maintained all cell types in apparently healthy condition during the first 2 weeks, but the organs then became necrotic. The AAB medium was distinct from the 3 previously described media, poorly supporting growth of even the non-germinal elements of the ovary. Few characteristic cells of any type were apparent after 2 weeks on this medium. Tyrode's solution alone failed to maintain cells of the ovary, although slight proliferation of fibroblasts occurred.

Cells of cultured testes were in better condition than those of the ovaries, and appeared essentially normal when grown on T.C. 199 or T.C. 1066, and also during the first 2 weeks on White's solution. The only group of testes cultured on AAB medium became necrotic prior to 28th day.

For maintenance of auxocytes 2 weeks or less, White's solution is most adequate; the other nutrient solutions in order of effectiveness were T.C. 1066, T.C. 199, AAB, and Tyrode's. For maintenance of young germ cells and non-germinal cells for longer times, media in order of decreasing effectiveness were T.C. 1066, T.C. 199, White's, AAB, and Tyrode's solution (Table I).

*Discussion.* These results indicate that media for growing mammalian and avian tissues will support amphibian gonads as organs for brief periods of time and maintain certain types of cells and tissues for a much longer time. However, none of the nutrient solutions was entirely adequate for maintenance of amphibian ovary or testis. The fact that ovaries, in general, were not as well maintained as testes might be due to inhibitory effects by products of cytolysis from the auxocytes. It appears that for the study of gonad differentiation which in amphibians may cover a relatively long period of time, of the used

TABLE I. Cellular Changes in Gonads on Synthetic Media for Varying Periods of Time.

| Media | Days in culture | Explants of ovaries | Germ cells: Auxocytes | Germ cells: Immature | Non-germinal cells | Testes | Germ cells | Non-germinal cells |
|---|---|---|---|---|---|---|---|---|
| T.C. 199 | 4 | 12 | Early stages of cytolysis | Normal | Increase in No. of follicular cells | 1 | Normal | Increase in No. |
| | 15 | 42 | Much cytolysis | Few | *Idem* | 3 | Present in only 1 gonad | *Idem* |
| | 20 | 7 | Remnants only | Very few | " | 0 | | |
| T.C. 1066 | 5 | 17 | Cytolysis of few cells | Normal | Slight increase in No. | 4 | Normal | Many mitoses |
| | 10 | 48 | 25% cytolysis | " | Increase in No. and size of cells | 11 | " | *Idem* |
| | 20 | 84 | 50-75% cytolysis | " | *Idem* | 18 | " | " |
| | 23 | 29 | *Idem* | " | Granulosa cells fill area around degenerating auxocytes | 0 | | |
| White's | 10 | 3 | Normal | " | Slight increase in No. | 5 | Normal | Slight increase in No. |
| | 13 | 2 | Cytolysis of few cells | " | Few mitoses | 6 | " | Normal |
| | 15 | 3 | Cytolysis of peripheral cells | Necrotic | No increase in No. | 3 | Few apparent | Some necrosis |
| | 25 | 9 | Cells necrotic | " | Necrotic | 2 | Necrotic | Necrotic |
| AAB | 6 | 6 | 50% cytolysis | Normal | Normal | 0 | | |
| | 16 | 17 | Marked cytolysis | Necrotic | Some normal | 0 | | |
| | 28 | 24 | Necrotic | " | Normal cells rare | 3 | Necrotic | Necrotic |
| Tyrode's | 16 | 17 | Marked cytolysis | " | *Idem* | 0 | | |

media, T.C. 1066 is most nearly adequate, while White's solution may serve for brief studies. It seems, however, that additions to or modifications of these media will be necessary for complete maintenance and differentiation of amphibian gonads *in vitro*.

*Summary.* Ovaries and testes of second-year larvae of *Rana catesbeiana* were cultured on synthetic media for varying periods of time up to 28 days. The organs were not as well maintained as on media from natural sources, but proliferation of non-germinal cells and young germ cells occurred on some of the tested media.

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## Effect of Growth Hormone on Plasma Unesterified Fatty Acid Levels of Hypophysectomized Rats. (24173)

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Studies by Greenbaum *et al.*(1,2,3), Weil (4) and Curry(5) and others have shown that growth hormone effects mobilization of lipids from depots and probable utilization of these lipids as a primary source of energy for the body. The studies of Greenbaum(1) suggest that lipid mobilization and utilization are the probable mechanisms by which growth hormone effects positive nitrogen balance and hence growth. Russell(6) suggests that the "protein sparing" effect of growth hormone might be enhanced by furnishing large amounts of fat to the diet. Gordon(7,8) and Dole(9) postulate that unesterified fatty acid (UFA) is liberated from fat depots when other sources of calories are decreasing or are not available. This study relates the effect of growth hormone on plasma unesterified fatty acid levels of hypophysectomized rats. By using hypophysectomized animals it is possible to obviate the effect of endogenous growth hormone.

*Methods.* Female Sprague-Dawley rats hypophysectomized at 150 g (Exp. I and II) and 250 g (Exp. III) were obtained from Hormone Assay Inc., Chicago, Ill. Before the studies were undertaken the animals were kept on rat-chow plus meat supplement diet for 10-14 days post hypophysectomy. Animals whose body weight had plateaued were considered adequately hypophysectomized. On the morning the experimental animals were injected intraperitoneally with 4 mg of growth hormone [Somar-A (Armour R-50109) courtesy Endocrinology Study Section, USPHS] in 0.5 ml of distilled water adjusted to pH 9.5. Control rats in Exp. I were injected with 0.5 ml of distilled water adjusted to pH 9.5. In Exp. II and III one group of animals was injected with 4 mg of inactivated growth hormone in 0.5 ml distilled water adjusted to pH 9.5. The growth hormone was inactivated by autoclaving the solution at 18 lb, 123°C for 1 hour. The other group in these experiments was injected with an active growth hormone preparation which was from the same lot as the inactivated preparation. After injection, animals were placed in individual cages and provided *ad libitum* with drinking solution containing 10% dextrose in 0.5 normal saline. The animals were sacrificed by decapitation 8 hours after injection. This time interval was demonstrated by preliminary studies with fasted rats to be the time of most significant difference of UFA levels between experimental and control animals.

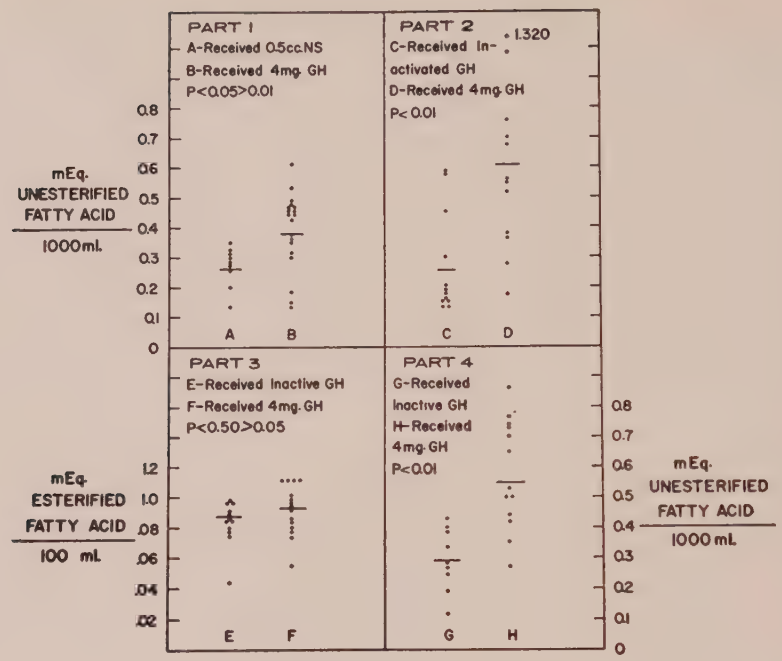


FIG. 1.

The decapitated animals were bled into centrifuge tubes containing Wintrobe's oxalate as anticoagulant. Unesterified fatty acids were determined by the method of Gordon(6) and esterified fatty acids by the method of Hack(10). A minimum of 1 ml of plasma was required for accurate determination of unesterified fatty acid levels.

*Results.* In Exp. I animals were injected with 4 mg of growth hormone, while controls received distilled water. The data of Fig. 1, Part 1 show that there was a significant difference in the plasma unesterified fatty acid levels between the 2 groups at the end of 8 hours. The mean of the experimental group was 0.392 meq UFA/1000 ml, while that of the control group was 0.265 meq UFA/1000 ml. This difference was significant at the 5% level but not at 1%. Exp. II was planned to determine the effect of injection of protein on serum unesterified fatty acid versus the effect of growth hormone injection. Therefore, in this experiment, 1 group of animals received inactivated growth hormone while the other received an identical amount of an active preparation. As shown in Fig. 1, Part 2, at the end of 8 hours the mean of the group receiving the active preparation was 0.605 meq UFA/1000 ml while that of the group receiving the inactive preparation was 0.258 meq UFA/1000 ml. This difference was significant at the 1% level. Exp. III was then planned to determine the effect of growth hormone on levels of serum esterified fatty acids and the relationship of these to serum levels of unesterified fatty acid. It was technically impossible to measure these levels sequentially. Fig. 1, Part 3 shows that the mean plasma esterified fatty acid concentration of the group receiving active growth hormone was 0.93 meq/100 ml after 8 hours, while that of the group receiving the inactivated preparation was 0.88 meq/100 ml. This difference is not significant at the 20% level. On the other hand, as can be seen from Fig. 1, Part 4, the mean of the unesterified fatty acid levels of the group receiving the active preparation was 0.566 meq UFA/1000 ml and that of the group receiving the inactive preparation was 0.294 meq UFA/1000 ml. This difference is significant at the 1% level.

*Discussion.* Our data would indicate that growth hormone has a definite effect on mo-

bilization of unesterified fatty acids in the hypophysectomized rat. Since the work of Gordon(7,8) and Dole(9) in humans indicates that with carbohydrate feeding the unesterified fatty acid level in the plasma decreases, it is of great interest to note that growth hormone caused an increase in plasma unesterified fatty acid in animals receiving carbohydrate *ad libitum*. The time required for this action to manifest itself (8 hours) correlates well with Greenbaum's(2) work in which a maximal increase in plasma and hepatic lipids occurred 6 hours after growth hormone injection. Weil's(11) work revealed a doubling of liver fat 7 hours post injection of 100 mg growth hormone.

At the present time the mechanism of growth hormone's mobilization of unesterified fatty acids from fat depots can be only speculated upon.

Release or formation of unesterified fatty acid following growth hormone administration could well provide an immediate source of energy, and thereby contribute to the protein anabolism seen after growth hormone administration. This phenomenon is well demonstrated by the studies of Greenbaum(1) and Beaton and Curry(4) who found that administration of growth hormone produced marked decrease in body adipose tissue and concomitant increase in body protein. The source of this unesterified fatty acid appears to be from adipose tissue according to studies by Gordon *et al.*(7). The observation has been made that serum esterified fatty acids are not altered by growth hormone administration and this may indicate that the unesterified fatty acid rise is derived *via* a different metabolic pathway.

*Summary.* Injection of growth hormone into hypophysectomized rats causes a significant rise in concentration of plasma unesterified fatty acids after an 8 hour interval. This rise in unesterified fatty acids is not associated with a concomitant rise in esterified fatty acid. Injection of inactivated growth hormone does not cause an increase in plasma unesterified fatty acid levels. It is postulated that this rise in unesterified fatty acids may provide an important source of energy for the known protein anabolic effect of growth hormone.

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## Occurrence of Fibrinolytic Activity Following Administration of Nicotinic Acid.* (24174)

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*In vivo* activation of fibrinolytic substances in blood has been accomplished by a variety of enzymes and extracts prepared from urine, plasma and tissues(1,2,3). This report describes a consistent observation of fibrinolytic activity in man following intravenous injection of a relatively simple substance, nicotinic acid. The observation was made during a

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TABLE I. Effect of I.V. Nicotinic Acid (1.0 mg/K) in Man on Clotting and Fibrinolytic Activity. (75 mg as sodium salt in 7.5 ml inj. over a 4 min. period.)

| Specimen | Time after completion of inj., min. | Prothrombin time (sec.) Whole plasma | Prothrombin time (sec.) Diluted (12.5%) plasma | Recalcification time, sec. | Residual thrombin activity,* sec.(7) | Plasma lysis† In 2 hr | Plasma lysis† In 24 hr | Coagulograph amplitude (mm) Maximum | Coagulograph amplitude (mm) At 2 hr |
|---|---|---|---|---|---|---|---|---|---|
| A | Control | 14.5 | 28.5 | 63.0 | 12.5 | 0 | 0 | 53 | 53 |
| B | 0 | 14.5‡ | 28.0 | 85.5 | 12.0 | 0 | 0 | 51 | 51 |
| C | 1 | | | | | 0 | 0 | 36 | 36 |
| D | 2½ | | | | | 0 | 0 | 36 | 35 |
| E | 4 | | | | | 0 | + | 31 | 29 |
| F | 6½ | | | | | ± | ++ | 18 | 3 |
| G | 10 | 15.0‡ | 29.0 | 50.0 | 12.0 | + | ++ | 35 | 3 |
| H | 13 | | | | | ++ | ++ | 29 | 1 |
| I | 16 | | | | | ± | ++ | 23 | 23 |
| J | 20 | 15.0‡ | 30.5 | 47.0 | 11.0 | 0 | ++ | 44 | 43§ |
| K | 35 | 15.0 | 31.5 | 63.0 | 13.5 | 0 | ++ | 33 | 33§ |
| L | Control + 50 mg/l nicotinic acid | 14.5 | 30.0 | 58.5 | 10.5 | 0 | 0 | 39 | 39 |

* By the technic used, 10 to 16 sec. is normal range of residual thrombin activity, with longer times reflecting increased antithrombin activity, and shorter times decreased antithrombin activity(7).

† 0 = no lysis; ± = minimal lysis; + = moderate lysis; ++ = complete lysis.

‡ Clots which formed were noted to have a fragile quality when lifted with a wire loop, although the endpoint of clotting test was clear.

§ Markedly reduced 24 hr later.

study of the influence of vasodilator drugs on the clotting mechanism.

*Results.* Table I describes the results of a typical experiment. Plasma specimens obtained 4 to 35 minutes after intravenous injection of 1 mg/kg of nicotinic acid in a 67-year-old hypertensive female over 4 minutes, showed evidence of fibrinolytic activity both by direct observation of the recalcified specimen in a test tube at 37°C, and, more quantitatively, by the coagulograph ("Thrombelastograph" of Hartert(4)) instrument. Although the patient noted the nicotinic acid "flush" primarily during injection, the specimens drawn up to 4 minutes after completion of injection showed little or no fibrinolytic activity. A decrease in clot firmness was noted one minute after completion of injection, with definite fibrinolysis in the specimen drawn 4 minutes after injection. Maximum fibrinolytic activity occurred in 10 to 15 minutes. Clotting time reactions were not significantly altered.

Fig. 1 presents the coagulograph patterns obtained in this experiment. The vertical distance between pairs of lines recorded for each specimen is a function of the firmness of the clot at any given moment(5,6). The control specimen (A) demonstrates the typical normal pattern of recalcified human citrated plasma. A single straight line is recorded prior to clot formation. With increasing clot firmness, a pair of diverging lines are recorded

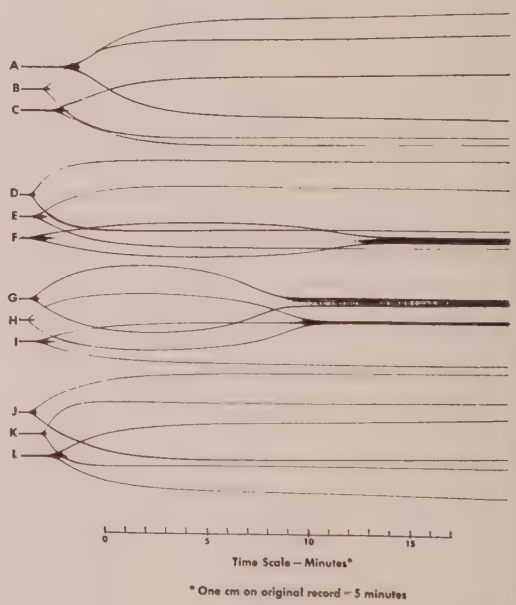


FIG. 1.

until maximum firmness is developed, at which time the lines become parallel. If lysis ensues, the lines begin to converge and merge into a single line when lysis is essentially complete. The pattern of lysis following nicotinic acid in this patient resembles that seen with lysis induced by material extracted from swine plasma(5) in contrast to the more gradual lysis seen with streptokinase activation. Specimen (L), which represents control plasma to which 50 mg/L of nicotinic acid had been added *in vitro,* shows no evidence of lysis. In other experiments, *in vitro* addition of nicotinic acid in concentrations ranging from 0.05 to 500 mg/liter plasma failed to influence fibrinolytic activity or clot firmness. In contrast, intravenous administration of nicotinic acid to 10 human subjects in doses of 1 to 3 mg/K induced fibrinolytic activity in all 10 instances. In this respect, the development of fibrinolytic activity by nicotinic acid *in vivo,* but not *in vitro,* resembles the activation of clearing factor by heparin.

*Conclusion.* It is concluded that a relatively simple non-enzymatic substance, nicotinic acid, can induce fibrinolytic activity *in vivo.*

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## Keratinization *in vitro* of chorionic Epithelium of the Chick Embryo.* (24175)

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The chorionic epithelium (CE) of the chick embryo forms the outer surface of the chorio-allantoic membrane (CAM). It consists of a single layer of small, attenuated cells of ectodermal origin, distally apposed to the shell-membrane and closely associated with blood vessels in the underlying mesenchyme in a manner characteristic of a respiratory epithelium (Fig. 1). Its basic structure is essentially retained also throughout later phases of embryonic development (Fig. 2), when capillaries penetrate through the CE to form a dense network on its surface. This chorio-vascular respiratory system continues to evolve until time of hatching when this structure, together with the rest of the CAM, is discarded with the shell. In its normal course of development, the CE shows no overt indications of morphogenetic propensities other than those which it displays in connection with its role as a respiratory epithelium; the possibility that its cells might possess additional potentialities which they would display under appropriate conditions, has not been adequately explored. In connection with another study, an attempt was made to maintain CE in organ culture in its original attenuated form. It was found, however, that the explants rapidly underwent striking metaplastic changes, during which they acquired new structural and functional characteristics.

*Material and methods.* Small fragments of CAM were excised from 8-day embryos, rinsed thoroughly in Tyrode's solution, then stretched on small squares of rayon-acetate net(1), the CE facing up, and cultured by

* This study was aided by a grant from the Smith, Kline and French Fn.

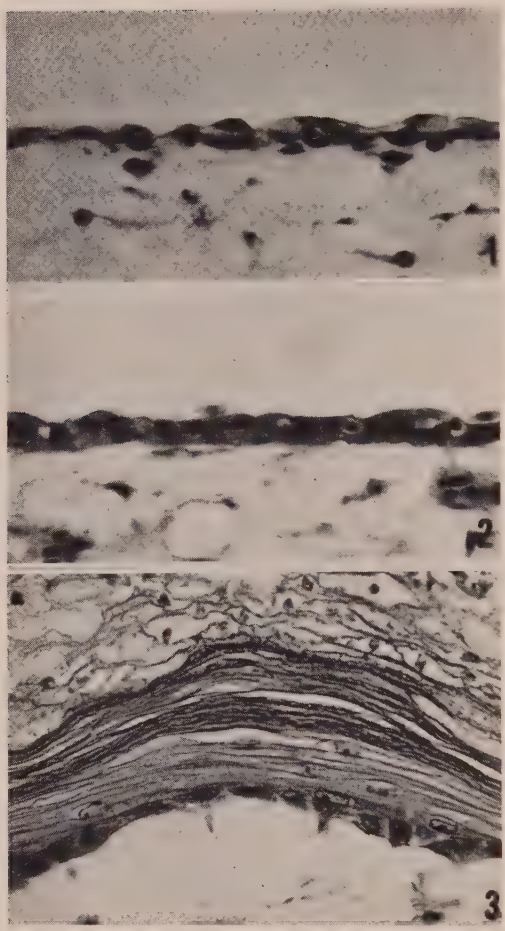

FIG. 1. Chorionic epithelium of 8-day chick embryo. Section through a control fragment of material at time of explantation, showing light staining elongated chorionic cells and dark, smaller endothelial and connective tissue cells. Ehrlich's hematoxylin and Biebrich's scarlet; × 400.

FIG. 2. Chorionic epithelium of an 18-day embryo. Chorionic cells and interspersed capillaries, endothelial and blood cells. E. h. and B. s. × 400.

FIG. 3. An 8-day culture of chorionic epithelium, showing keratogenic metaplasia as indicated by outward proliferation of cells, squamous changes and layers of keratin. E. h. and B. s. × 400.

Fell's watch-glass method(2). The clot was made up of 3 parts of fowl plasma and one part of chick embryo extract (11-day embryos). The culture dishes were kept in sealed containers, under conditions of saturation humidity, with ample supply of air. The explants were transferred to a fresh medium every 2 days and maintained in culture for 8 days. They were then fixed in Zenker's fixative, processed according to Shaffer's procedure(1), sectioned and, either stained with hematoxylin and Biebrich's scarlet, or examined in polarized light for birefringence.

*Results.* During the first 4 days of cultivation the explants, examined in the living state, showed few noticeable changes. From the fifth day on they became progressively more opaque, due to appearance on their surface of delicate sheets of light-reflecting material, wrinkled and parchment-like in texture. Scraped off fragments of this material were examined in polarized light and found intensely birefringent. In histological sections the CE changed from a membrane one-cell in thickness into a multilayered epithelial structure in an advanced stage of keratinization (Fig. 3). The *basal layer* of this epithelium corresponded in position to that of the original CE and consisted of one row of cuboidal or somewhat elongated cells. The *intermediate layer* comprised several rows of flattened cells, showing distally progressive cytoplasmic and nuclear changes characteristic of squamous keratinizing epithelia; accordingly, there was also an outwardly increasing affinity for Biebrich's scarlet. In polarized light, the upper strata of this layer showed scattered birefringence. The *outer layer* consisted of rows of keratin which stained intensely with Biebrich's scarlet and were strongly birefringent. This keratinized material was evidently largely responsible for the characteristic sheen and parchment-like appearance of the surface of living cultures. On top of the keratin there was usually a mass of deformed cellular fragments and hydrated or lysed cells or fibers.

In addition to the outward proliferation and keratinization of the CE cells, there was also an inward directed formative activity. Keratinized cysts, vesicles and epithelial pearls were occasionally present beneath the basal layer, within the underlying mesenchyme. The frequency of appearance of these formations could be markedly increased by lightly injuring the freshly explanted CE with a fine needle and pushing the dislodged cells into the mesenchyme.

*Discussion.* The recorded observations have shown that the CE of the chick em-

bryo, when grown *in vitro* for several days, became altered from a simple respiratory membrane into a multilayered keratogenic tissue. The precise identity of the stimuli directly involved in causing this metaplasia remains to be examined. One possible assumption is presence in the culture environment of metabolic requirements, not available to the CE *in ovo* but essential for manifestation of the keratogenic properties of the CE cells. Alternatively, the existence *in ovo* of inhibitory conditions, affecting the CE so as to prevent it from expressing its keratogenic potentialities, should be considered.

Clarification of these and other possibilities, might also relate to the general problem of keratinization. The CE, in being a normally non-keratogenic tissue, appears to be a particularly suitable material for studying processes and events by which cells may acquire capacities for rapid and voluminous production of keratin; it thus provides a useful source of comparison with the properties of skin. In view of the mucogenic metaplasia of embryonic skin demonstrated by Fell and Mellanby(2) and the role of Vit. A in this process(3), the reaction of the CE to this vitamin might be of interest. In this connection, one should also like to know if conditions might be found which would cause the CE to undergo *in ovo* keratogenic changes similar to those displayed in culture. These questions are being presently investigated.

*Summary.* A keratogenic metaplasia *in vitro* of chorionic epithelium of the chick embryo is described. Explants of chorionic epithelium from 8-day embryos, cultured for 8 days on a plasma clot, became altered from an attenuated, one-cell-thick respiratory membrane into a multilayered, squamous epithelium in advanced phases of keratinization.

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## Osteolathyrogenic Action of Mercaptoethylamine and of Cystamine.* (24176)

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Beta-mercaptoethylamine, when fed to weanling rats, was found to produce gross osteolathyric lesions similar to those produced by diets containing sweet peas or β-aminopropionitrile (BAPN)(1). Ponseti *et al.*(2) were unable to confirm this finding. Results of studies on wound healing(3) and of studies using pregnant rats(4) appear to be in agreement with the negative report of Ponseti *et al.* We have now repeatedly produced osteolathyric-like lesions not only with different lots of mercaptoethylamine but with closely-related cystamine as well.

*Methods.* Beta-mercaptoethylamine was fed as the hydrochloride; cystamine as the dihydrochloride. Three different lots of β-mercaptoethylamine · HCl were purchased from California Fn. for Biochemical Research over a period of 18 months and were stored in vacuum desiccator. Iodometric titrations for –SH as well as chloride determinations indicated a purity of 96% or more for each of the lots. Cystamine 2HCl was purchased from Nutritional Biochemicals Corp. Iodometric determination of –S–S– following reduction(5) and chloride determination indicated a purity of 99%. These supplements were incorporated into finely milled Rockland Rat Stock

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Diet and fed to male albino rats of Sprague-Dawley strain weighing 50-62 g. Selected rats were sacrificed after 3 weeks or more on these diets. The entire aorta and one hind extremity were fixed in 10% buffered formalin. The knee regions (distal femur, joint proper, proximal tibia and fibula) were decalcified in equal parts of 10% formic acid and 20% sodium citrate. Aortas were cut into 8 segments of equal length, dehydrated, cleared and embedded in paraffin. Six to 8 $\mu$ sections were stained with hematoxylin and eosin, and with Hornowski's combined elastic and Van Gieson stains.

*Results.* Because of the severe growth-depressing effect of *mercaptoethylamine · HCl,* it was found advisable not to use rats too light in weight. Best results were obtained with rats weighing about 60 g and fed diets containing about 0.45% of the hydrochloride. Development of osseous lesions in different rats was not as uniform as when salts of BAPN were fed, but all rats which survived sufficiently long showed skeletal changes. Gross skeletal effects are shown in Fig. 1. No gross lesions of the aorta were seen in any of these animals. Perhaps because of its greater stability, osteolathyrogenic effects were somewhat easier to demonstrate with *cystamine · 2HCl.* Small differences in dietary levels, however, gave rise to marked differences in response. On a diet containing 0.2% cystamine · 2HCl, rats weighing 50-60 g gained weight at a rate only slightly less than that of control rats receiving the unsupplemented diet. The only gross symptom observed over a period of 7 weeks was a slight kyphosis detectable by careful palpation or by roentgenological examination. Rats receiving 0.5% cystamine · 2HCl exhibited an immediate loss in weight and some died within one week on the diet. Those that survived 12-14 days or longer had spinal curvatures, prominent, palpable exostoses on the mandibles, and showed marked skeletal lesions both grossly (Fig. 1) and microscopically. Only 1 of 8 rats survived beyond 19 days on this level. On a 0.4% level, 1 rat in 6 survived beyond 24 days. At 0.3%, all rats survived beyond 5 weeks at which time some of animals were sacrificed. All rats on 0.3% and 0.4% levels developed gross skeletal changes typical of osteolathyrism, palpable skeletal lesions becoming detectable in about 3 weeks and 2 weeks respectively at these levels.

*Histopathologically,* similar aortic and skeletal lesions were observed in both groups of animals. Although no gross aneurysms were seen in aortas of any rats fed mercaptoethylamine or cystamine, aortas of animals in both groups showed swelling and degeneration of tissues between elastic fibers of the media. In most animals the elastic fibers remained intact. Some showed multiple small areas of destruction involving the outer ⅔ of the media and sparing the inner portion. All elastic fibers were destroyed in these areas with considerable replacement by collagen. These changes (Fig. 2) were similar to elastolytic changes observed in the aortic media of rats and mice receiving sweet peas or BAPN(6,7,8,9). In the aorta of one rat receiving mercaptoethylamine · HCl, calcium deposition had taken place in areas of medial destruction. No evidence of dissecting hemorrhage or aneurysm was seen in any of the animals.

Periosteal lesions were observed in all specimens from both groups. These showed: 1, fibroblasts with collagen formation, 2, osteoblasts with osteoid formation, 3, chondroblasts with cartilage formation, 4, basophilic granules or globules in new bone and cartilage matrix (Fig. 3). Similar changes have been produced by sweet peas, BAPN, and aminoacetonitrile(10,11,12,13). Extensive recent hemorrhage in the proliferating periosteum was seen in one animal receiving cystamine · HCl. Periosteal hemorrhage has been reported in rats fed sweet peas(14).

Changes observed in the epiphyseal cartilage were: 1) irregular thickening and broadening of the plate, 2) balloon degeneration of cartilage cells, 3) abnormal proliferation of cartilage cells, 4) disruption of parallel rows of cartilage cells to form irregular groups or masses, 5) fibrillar degeneration of cartilage matrix, 6) areas of complete destruction of both cells and matrix with space or cleft for

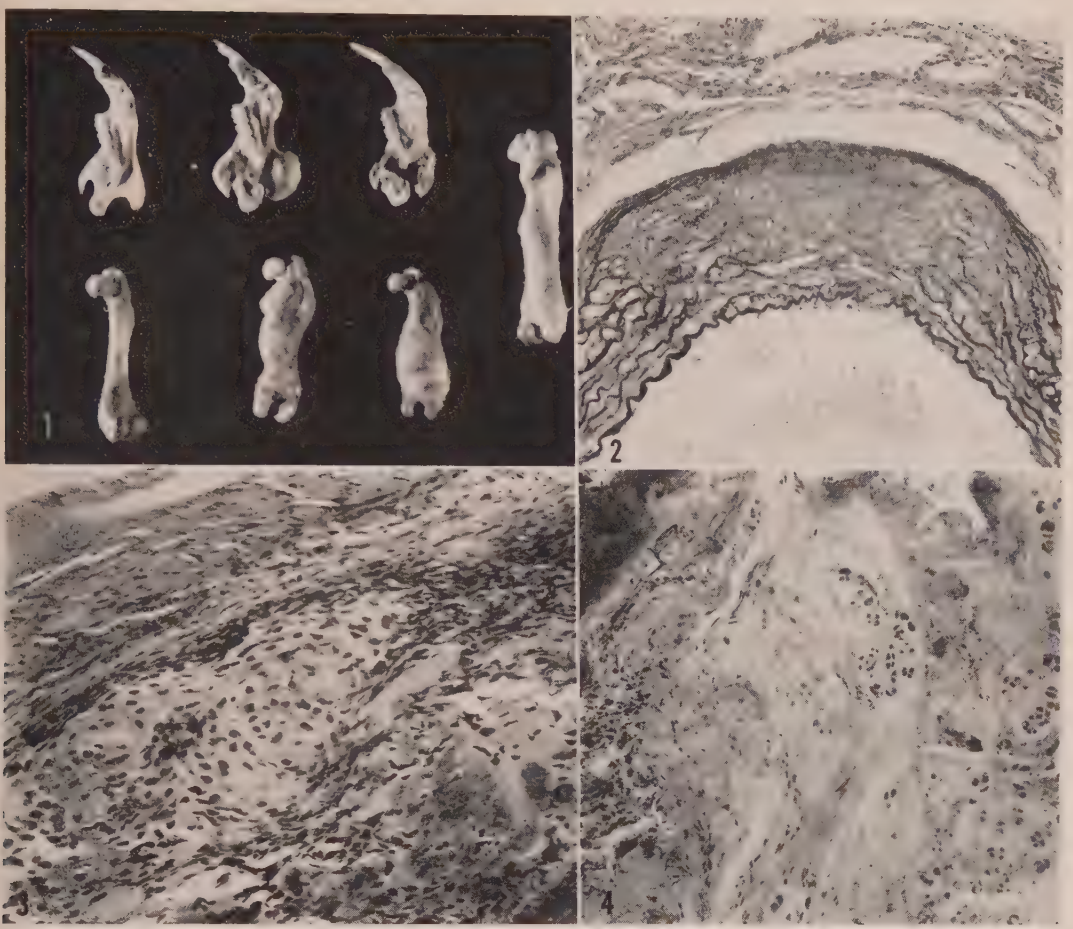

FIG. 1. Rat femurs and mandibles, left to right: normal; .45% β-mercaptoethylamine · HCl, 22 days; .5% cystamine · 2HCl, 22 days; .45% β-mercaptoethylamine, 84 days.
FIG. 2. Aorta of rat receiving .4% mercaptoethylamine · HCl for 37 days. × 200.
FIG. 3. Periosteal proliferation in same rat as Fig. 2. × 300.
FIG. 4. Epiphyseal cartilage in rat receiving .5% cystamine · 2HCl for 22 days. × 300.

mation (Fig. 4). Sweet peas, BAPN and aminoacetonitrile produce similar changes (11,12,15).

Specimens from all experimental rats examined showed fatty transformation of bone marrow. This was generally more evident in the epiphysis than the metaphysis. Fatty transformation of bone marrow has been described as an occasional finding in osteolathyrism induced by feeding sweet peas(14). In osteolathyrism resulting from ingestion of semicarbazide, fatty transformation of bone marrow is even more marked than when mercaptoethylamine or cystamine is fed (unpublished).

In the joint proper, no definite changes in synovial membranes or articular cartilage were observed.

No hernias were seen. However, the incidence of hernias in hundreds of rats receiving sweet peas or BAPN has been well under 1% in contrast to its high incidence in other laboratories(6,16).

*Discussion.* The fact that gross skeletal lesions and histopathological changes in periosteum, epiphyseal cartilage, and aortic media of rats fed β-mercaptoethylamine · HCl or cystamine · 2HCl seem identical to lesions observed in rats fed sweet pea toxin (BAPN) appears to indicate that the syndromes are identical. The use of the term *osteolathyrism* (13) for these conditions therefore seems jus-

tified. Whether these different substances affect the same specific metabolic reaction or act at different points in the same metabolic sequence remains to be determined. There are, however, differences between manifestations of mercaptoethylamine or cystamine toxicity and those produced by osteolathyrogenic nitriles. The former have a much narrower range of osteolathyrogenic activity between lethal and totally ineffective levels. Aortic lesions were also much less severe when compared on the basis of severity of bone lesions. In fact, no evidence of dissection or aneurysm formation was observed in any animals.

Since β-mercaptoethylamine (cysteamine) and cystamine are readily interconvertible by oxidation and reduction respectively, it is likely that the *in vivo* toxic compound is the same in both instances. The more consistent results obtained with cystamine are probably due to its greater stability in the diet.

In view of our findings, the results of toxicity experiments reported by Bacq(17) are entirely inexplicable. He fed cystamine · 2HCl at levels up to 1% for periods up to 2 months. His rats grew well and showed no symptoms of lathyrism. Although his rats were somewhat larger than ours, our experience with cystamine would lead us to expect a high mortality and 100% incidence of osteolathyrism in rats surviving as long as 2 weeks.

*Summary.* The osteolathyrogenic action of β-mercaptoethylamine (cysteamine), when fed to rats as the hydrochloride, was confirmed. Cystamine was found to have similar properties. Gross and microscopic skeletal changes were similar to those produced by osteolathyrogenic nitriles. Aortic lesions were much less severe than when β-aminopropionitrile is fed, but medial swelling and lysis of elastic fibers were observed. These substances showed a narrow range of osteolathyrogenic activity, a dietary level of 0.2% cystamine · 2HCl causing only very slight spinal curvatures, while a level of 0.5% was rapidly lethal to most animals.

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# Bruised Tissue IV. Effect of Streptokinase and Trypsin on Healing.* (24177)

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It has been shown that each of 3 successive bruises in animals healed more rapidly than did the preceding one(1). The factor or factors responsible for this accelerated healing are, at least in part, present in whole blood from a previously bruised animal (rabbit), and may be passively transferred to a normal animal by transfusion. No single substance has been isolated which would completely account for accelerated healing of a secondary wound. Engley, *et al.*,(2) have suggested that an unidentified humoral factor may be involved while Zamencik, *et al.*,(3) established the existence of an enzyme (aminoexopeptidase) in lymph and serum of dogs, and observed that levels of this enzyme rise abruptly in lymph draining an area of burned or traumatized tissue. Others(4-6) have reported the presence of proteolytic enzymes in damaged tissue. Sherry and Alkjaersig(7) described the beneficial effect of streptokinase for dissolution of intravascular thrombi and extravascular fibrinous exudates in dogs, while Gordon and Ablondi(8) reported that intravenous injection of this enzyme reduced the severity of inflammation experimentally produced in rabbits. Trypsin also has been shown effective in reducing experimentally induced edema(9). These enzymes have proven successful as anti-inflammatory agents by other investigators(10-15). The purpose of this study was to determine the influence of trypsin and a mixture of streptokinase and streptodornase on the biochemistry of healing of experimentally induced bruises in rabbits and cattle.

*Materials and methods. Bruising and sampling.* Rabbits (6-8 lb) and cattle (700-850 lb) were employed. Areas to be bruised and symmetrically located control areas were denuded. Procedures for inflicting bruises and for procuring tissue samples were previously described(16). Morphologic examination of bruised tissue, detection of tissue bilirubin as a reflection of hemoglobin degradation and conductivity measurements(1,17) served as criteria for the effectiveness of test enzymes in promoting the healing process. *Bilirubin.* Presence of bilirubin in bruised tissues(16) was detected using Fouché reagent, forming no color (–) in its absence and a light blue color in presence of 0.5-1.0 mg bilirubin/100 g tissues (indicated by +). This color turns to dark blue (++) and (+++) at a concentration of 1-2.5 mg bilirubin and to green (++++) above this concentration(17). This blue green color (probably biliverdin) is a reaction product of oxidation of bilirubin by ferric chloride in the trichloro-acetic acid solution (Fouché reagent). *Enzymes.* Trypsin† and streptokinase-streptodornase varidase‡ (SK-SD) enzyme preparations were used. Unless otherwise stated, these enzymes were introduced into the experimental animals 12 hours after bruising.

*Results. Effect of injecting trypsin and SK-SD on bruised tissue.* Working levels of test enzymes were established by injecting normal rabbits either intramuscularly or subcutaneously in graded amounts. The results indicated that a single injection of trypsin to 10 mg did not result in necrosis, while daily injections of 6 mg for 15 days resulted later in erythematous areas at injection sites. A single injection of SK-SD to 15,000 units

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† Paraenzyme, a sterile suspension of trypsin in sesame oil (Generously provided by National Drug Co., Phila., Pa.).

‡ A purified and concentrated combination of enzymes produced by a strain of streptococcus (Lederle Lab. Div. of American Cyanamid Co., Pearl River, N. Y.).

TABLE I. Effect of Trypsin and Streptokinase-Streptodornase (SK-SD) on Healing Time, Bilirubin Detection and Conductivity in Rabbit Bruises.

| No. of animals | Agent used | Healing time (days) | Bilirubin detection (hr) | Conductivity $\times 10^{-2}$ ($ohm^{-1}$ $cm^{-1}$) After 2 days | Conductivity $\times 10^{-2}$ ($ohm^{-1}$ $cm^{-1}$) After 5 days |
|---|---|---|---|---|---|
| 10 | 2 mg trypsin | 6.5 ± .2 | 52 ± 2.5 | 6.1 ± .3 | 2.95 ± .3 |
| 11 | 10,000 units (SK-SD) | 5.8 ± .2 | 48 ± 3.8 | 5.8 ± .3 | 2.9 ± .4 |
| 7 | 1 ml saline (control) | 8.0 ± .3 | 71 ± 2.5 | 5.9 ± .5 | 4.1 ± .3 |
| 5 | 1 ml sesame oil " | 8.2 ± .4 | 72 ± 3.0 | 5.4 ± .4 | 4.3 ± .3 |
| 7 | No treatment " | 7.9 ± .3 | 70 ± 3.0 | 5.7 ± .3 | 4.3 ± .4 |

failed to elicit any discernible effect. Twenty thousand units as a single injection, or daily administration of 10,000 units for 15 days, produced inflammatory manifestations.

Five groups of rabbits were bruised according to a standard procedure(16). Twelve hours later, animals in Group I were injected intramuscularly with 2 mg of trypsin at the site of the bruise; Group II animals received 10,000 units of SK-SD by the same route; rabbits in Group III were inoculated with 1 ml of physiologic saline; Group IV, 1 ml of sesame oil; and Group V animals served as untreated controls. Observations of the bruised areas continued for 9 days. At intervals animals were sacrificed; bruised and control areas were compared grossly; and the average resistances offered by these tissues were measured and conductivities calculated using the cell constant, as previously described(17). Samples of bruised and control tissues were immediately excised, scored and tested for the presence of bilirubin. The latter substance was apparently bound to protein, thus confirming the report by Stenhagen and Rideal(18).

As shown in Table I, injections of trypsin or SK-SD accelerates the healing process of bruised tissues as determined by gross examination. Average healing occurred in 6.5 days in rabbits which received trypsin, 5.8 days in animals treated with SK-SD, while controls (untreated, saline, or sesame oil) required approximately 8 days for complete repair. The first detection of bilirubin (an early indication of reparative processes) in each group paralleled the above pattern. Conductivity measurements after 2 days were of approximately the same magnitude, but after 5 days, values for enzyme-treated tissues approached those previously established for normal tissues. This experiment was repeated in part in cattle. In these animals concentrations of enzymes employed for treatment were increased to 10 mg of trypsin and 45,000 units of SK-SD. The results were similar to those described above.

*Enzyme injections at sites remote from bruises.* A number of rabbits, bruised singly over the gluteus muscle, were divided into 5 groups. Each animal in the first group was injected daily in the bruised muscle with 2 mg of trypsin; animals in the second group were treated in the same manner, except that daily injections of enzyme were introduced into a site remote from the bruise. The third and fourth groups received 10,000 units of SK-SD daily at the locus of the bruise and in a distant tissue, respectively. The fifth group of animals were untreated. Animals from each group were sacrificed after 52, 88, and 106 hours, and the same measurements were conducted as before (Table II). Bilirubin was detected in bruised tissue after 52 hours, in animals receiving the enzyme preparation at site of bruise but not in the control (untreated animals or animals receiving the enzyme at a site remote from bruise). After 88 and 106 hours, very little difference was noted among the various groups, either in quantity of bilirubin detected or in conductivity values. After 106 hours, evidence of more rapid repair was obtained from those animals treated with both enzyme preparations at sites of the bruises (conductivity values). Rate of healing in those rabbits injected in other than damaged tissues resembled that noted for untreated controls.

*Influence of ascorbic acid in healing.* Rabbits, bruised over the gluteus muscle, were

TABLE II. Effect of Site of Injecting Trypsin or SK-SD on Healing of Bruised Tissues in Rabbits. Three animals bruised per series.

| Treatment received (daily) | Bilirubin detection after | | | Conductivity × $10^{-2}$ ($ohm^{-1}$ $cm^{-1}$) | | |
|---|---|---|---|---|---|---|
| | 52 hr | 88 hr | 106 hr | 52 hr | 88 hr | 106 hr |
| 2 mg trypsin (at the site*) | + | ++++ | +++ | 6.0 ± .3 | 4.8 ± .3 | 3.3 ± .3 |
| *Idem* (at remote site†) | — | ++ | ++++ | 5.9 ± .2 | 5.0 ± .3 | 4.5 ± .3 |
| 10,000 units SK-SD (at the site) | + | +++ | +++ | 6.0 ± .3 | 4.9 ± .2 | 3.6 ± .3 |
| *Idem* (at remote site) | — | ++ | ++++ | 6.1 ± .3 | 5.2 ± .3 | 4.2 ± .3 |
| No treatment (control) | — | ++ | ++++ | 5.9 ± .2 | 5.1 ± .3 | 4.6 ± .3 |

* At the site of bruised tissue. † At site remote from bruised tissue.

TABLE III. Effect of Injecting Ascorbic Acid and Trypsin on Healing of Rabbit Bruises. Three animals bruised per series.

| Treatment received (daily) | Bilirubin detection | | Conductivity × $10^{-2}$ ($ohm^{-1}$ $cm^{-1}$) | |
|---|---|---|---|---|
| | After 52 hr | After 106 hr | After 52 hr | After 106 hr |
| 2 mg trypsin + .17 mg ascorbic acid | ++ | + | 6.2 ± .3 | 2.8 ± .2 |
| 2 mg trypsin | + | +++ | 6.0 ± .3 | 3.3 ± .3 |
| 0.17 mg ascorbic acid | + | +++ | 6.0 ± .3 | 5.0 ± .3 |
| No treatment (control) | — | ++++ | 5.9 ± .2 | 4.6 ± .3 |

grouped according to treatment to be rendered. Animals of the first group were injected daily with 2 mg of trypsin at the bruise site, as before. Each animal in the second group received the same quantity of trypsin as animals of first group, and, in addition, 0.17 mg of ascorbic acid contained in 0.5 ml of 1/15 M phosphate buffer (pH 7.4).§ Animals in the third group were injected with the ascorbic acid only, while the fourth group was retained as untreated controls. Animals from each group were sacrificed after 52 and 106 hours.

The results (Table III) indicate that bruised tissues of the group treated daily with the mixture of enzyme and ascorbic acid healed at a significantly more rapid rate than did tissue of animals in the remaining groups.

*Discussion.* The enhanced healing observed in bruised animals following treatment with trypsin or SK-SD may confirm the opinions of others regarding the mode of action of these agents(7-15). Most likely trypsin functions directly to accelerate proteolysis of damaged tissue, while the SK-SD activates plasminogen. Thus the normal body defense mechanisms are enhanced to function more efficiently.

Activation of proteolytic enzymes either artificially, as reported here, or as a natural phenomenon associated with tissue repair, may account for accelerated healing of successive bruises, provided critical levels of these enzymes exist at the time of bruising. Sherry and Alkjaersing(7) reported that trypsin and streptokinase are able to activate blood plasminogen to form plasmin, which is capable of splitting arginine and lysine esters as established by Troll and Sherry(19).

Some bruises did not respond to trypsin or streptokinase treatment and accordingly healed at a much slower rate. This may be due to the presence of a high level of antitrypsin or antistreptokinase. It is also possible that animals which did not respond to streptokinase had or have had a recent streptococcal infection and hence had a high titer of antibodies specific against the inoculated enzyme.

Müllertz(15) reported that failure of some animals to respond to the streptokinase treatment may be attributed to absence of plasminogen or to high level of antistreptokinase, a specific immune antibody developed during streptococcal infection or to a high level of antiprotease that occurs in rather high con-

§ The ascorbic acid solution was prepared immediately prior to administration to minimize oxidation.

centration in animal sera, or to a strict species specificity to streptokinase.

The precise role of ascorbic acid in repair processes is not known. The possibility exists that Vit. C may be utilized by injured tissues for synthesis of collagen(20), Lemberg, *et al.*,(21) reported that coupled oxidation may occur between hemoglobin and ascorbic acid for the formation of choleglobin. Bourne (22) established that the tensile strength of a healing wound in guinea-pig suffering from partial deficiency of ascorbic acid is considerably less than in normal animals.

*Summary.* 1. Rabbits previously bruised healed more rapidly following injections of trypsin and SK-SD than did their untreated counterparts. 2. Injections of enzyme preparations into tissue remote from the bruised areas did not result in accelerated healing. 3. Combination of Vit. C and trypsin was superior to trypsin alone in promoting tissue repair.

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## Effect of 6-Dimethylaminopurine-3-Amino-D-Ribose on Adenosine Triphosphate Formation in Yeast.* (24178)

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It has been demonstrated that 6-dimethylamino-purine-3-amino-d-ribose (Aminonucleoside) induces the nephrotic syndrome in rats (1,2). In an effort to obtain evidence of the mechanism of production of this experimental nephrosis, the effect of this compound on adenosine metabolism has been investigated. This study was prompted by reports which indicate that Aminonucleoside may interfere with some phase of purine metabolism. Agosin and Von Brand(3) and Hewitt, Gumble, Wallace and Williams(4,5) demonstrated that the trypanosomacidal action of the drug in infected mice could be blocked by simultaneous administration of various substituted purines. These observations, and similarity in chemical configuration of adenosine and Aminonucleoside, suggested the possibility of competitive inhibitory effect of Aminonucleoside upon adenosine metabolism. To test this hypothesis, an *in vitro* system known to metabolize adenosine was sought. Crude yeast fractions

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possess an enzyme, designated by Sable(6) as adenosine phosphokinase. In such fractions, a net production of adenosine triphosphate (ATP) from adenosine occurs.† Kornberg and Pricer(7) partially purified the yeast enzyme. Although 17 nucleosides were tested, substrate specificity was shown only for adenosine and 2-amino-adenosine. Aminonucleoside was not included in compounds studied. Caputto(8) subsequently found adenosine phosphokinase in animal tissue, namely kidney and liver, as well as in yeast. Studies of the animal enzyme indicated that adenosine in the presence of ATP was metabolized to adenosine-5¹-phosphate (AMP). Similar findings were obtained with yeast enzyme(7). It was therefore concluded that in crude yeast systems where ATP is formed from adenosine, the synthesis is stepwise from AMP. The subsequent steps involve formation of adenosine-diphosphate (ADP) by myokinase and of ATP by such enzymes as pyruvate phosphokinase. Our work is concerned with studies in a crude yeast system where the effect of Aminonucleoside on conversion of adenosine to ATP was investigated.

*Materials and methods. Materials*: ATP, triphosphopyridine nucleotide (TPN), glucose-6-phosphate dehydrogenase (Zwischenferment) and hexokinase were obtained from Sigma Chemical Co. The adenosine used was the product of Schwarz Laboratories. Dried brewer's yeast was supplied by Sigma Chemical Co. (Lot No. 24-605). We are indebted to Lederle Laboratories for 6-dimethyl-aminopurine-3-amino-d-ribose (Aminonucleoside). *Methods*: Incubations were carried out in Erlenmeyer flasks. The incubation mixture was as follows: 2.5 g of yeast and 0.05 ml toluene in 10 ml 0.1 *M* $Na_2HPO_4$ buffer, pH 7.0. By means of an electromagnetic device, stirring was continued throughout incubation period. Temperature was maintained at 25°C. Adenosine, the substrate, was added at final concentration of 37.0 $\mu$M/ml. Aminonucleoside, in concentrations of 37.0 to 185 $\mu$M/ml was tested in the absence and presence of adenosine. Aliquots of 0.1 ml were taken at 0 time and at 10 minute intervals over 60-120 minute incubation. Deproteinization of aliquots was accomplished in 10 ml of 10% trichloracetic acid (TCA). 1.0 ml aliquots of the centrifuged supernatant were then analyzed for inorganic phosphate(9) and acid-labile phosphate. Acid-labile phosphate was determined as that phosphate liberated in 10 minutes at 100°C in 1 *N* $H_2SO_4$. Measurements of ATP and other phosphate esters formed during incubation were obtained as follows: Reaction mixtures were cooled to 2°C in ice bath. 0.6 ml of 60% perchloric acid was added. The mixture was then filtered and precipitate washed with 2 ml portions of 3% perchloric acid. To combined filtrates were added 4 volumes of 95% ethanol. The precipitate was collected by centrifugation and taken up in 2.0 ml $H_2O$. The pH was then adjusted to 7.0. This material was then assayed spectrophotometrically for glucose-6-phosphate by reduction of TPN in the presence of Zwischenferment and hexokinase. Absence of glucose in each sample was ascertained by Somogyi-Nelson method(10). After completion of reaction, glucose was added and ATP determined, as described by Kornberg (11). Total phosphate was determined after ashing in 10 *N* $H_2SO_4$. Paper chromatographic separation of nucleotides and sugar phosphate esters was carried out on these extracts according to the methods of Krebs and Hems(12) and Bandursky and Axelrod(13), respectively.

*Results.* I. Inorganic phosphate uptake and acid labile phosphate formation. *Yeast and yeast plus adenosine.* Under incubation conditions described, in absence of added substrate a disappearance of inorganic phosphate (Pi) from the medium is noted. Maximum disappearance occurred after 60 minutes. During this period 14.9 $\mu$M/ml of acid-labile phosphate (Po) was formed. On addition of the substrate, adenosine, maximal uptake of Pi was increased and Po production was more than doubled (Table I, Exp. 1). Fig. 1 illustrates the time course of these changes.

*Yeast plus Aminonucleoside.* In contrast to the effect produced by adenosine, addition of equimolar amounts of Aminonucleoside as

† Personal communication from Sigma Chemical Co.

substrate produced only a small increase in Pi uptake over that noted in the absence of substrate. Further, an actual decrease in the amount of Po formed was noted. (Table I, Exp. II).

*Yeast, adenosine and Aminonucleoside.* In the presence of adenosine, addition of Aminonucleoside exerted an inhibitory effect on Pi uptake and Po formation, the magnitude of which was dependent upon concentration of Aminonucleoside (Table I, Exp. III). When Aminonucleoside and adenosine were present in equal concentrations, maximal amount of Po formed was equal to that formed in the absence of the Aminonucleoside, although there was a significant time lag (Fig. 1). When concentration of Aminonucleoside was increased to twice that of adenosine, Po formation declined from a control value of 31.0 $\mu$M/ml to 6.8 $\mu$M/ml and Pi uptake decreased in a like manner. Inhibition of Po formation became complete as concentration of Aminonucleoside was increased to 5 times that of adenosine.

II. ATP Production. To identify the Po or acid-labile phosphate compounds formed in the yeast incubation mixture, the perchloric acid-ethanol procedure was carried out on mixtures indicated in Table II. These extracts were then analyzed by paper chromatography (12). ATP was easily seen in all reaction mixtures except that containing 0.1875 *M* Aminonucleoside, that is, a 5:1 ratio of Aminonucleoside to adenosine. The adenosine incubation mixture showed the largest ATP spot. No other nucleotides were identified. To obtain more quantitative data, ATP was enzymatically assayed (Table II). These results are in accord with labile phosphate formation experiments in the following respects: (1) addition of adenosine to yeast resulted in a significant increase in ATP production; (2) a very small increase in ATP was noted upon addition of Aminonucleoside to yeast; and (3) in the presence of adenosine and high concentrations of Aminonucleoside, virtually complete inhibition of ATP production was observed.

III. Phosphate analysis. A representative group of ethanol precipitates was chosen for complete phosphate analysis (Table III). For each $\mu$mole of ATP one should find 2 $\mu$moles of acid-labile phosphate; however, it was observed that in each precipitate this ratio was greater than 2:1. (The exception of

TABLE I. Effect of Aminonucleoside on Maximal Acid Labile Phosphate Production and Inorganic Phosphate Uptake.

| Exp. | | Change in acid labile phosphate (Po), $\mu$M/ml | Change in inorganic phosphate (Pi), $\mu$M/ml |
|---|---|---|---|
| I | Control | +14.9 (2)* | −70.4 (2) |
| | Exp. | +40.7 (2) | −81.5 (2) |
| II | Control | +13.2 (2) | −58.1 (2) |
| | Exp. | + 9.4 (2) | −64.0 (2) |
| III | Control | +31.0 (6) | −86.3 (6) |
| | Exp. 1 | +34.5 (2) | −82.6 (2) |
| | 2 | + 6.8 (2) | −27.4 (2) |
| | 3 | − 5.5 (2) | + 1.3 (2) |

Exp. I Control: Yeast only
Exp.: " + adenosine 0.0375 M

Exp. II Control: " only
Exp.: " + aminonucleoside 0.0375 M

Exp. III Control: Yeast + adenosine 0.0375 M
Exp. 1: *Idem* + aminonucleoside 0.0375 M
2: " + " 0.0750 M
3: " + " 0.1875 M

All mixtures contained phosphate buffer, toluene and yeast as described. Values represent results obtained at point of maximal Pi uptake and Po formation. Control values were obtained simultaneously with exp. values.

* No. of experiments.

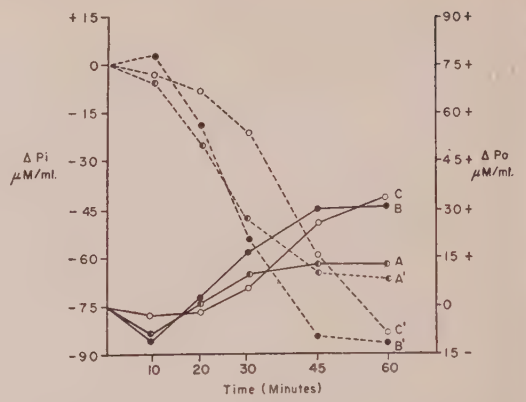


FIG. 1. All experimental mixtures contained phosphate buffer, toluene, and yeast as described above. ---- represents uptake of inorganic phosphate and ——— formation of acid labile phosphate. A and A′ show results of incubating yeast alone; B and B′, the results when 0.0375 M adenosine is added and C and C′, the findings when 0.0375 M adenosine plus 0.0375 M aminonucleoside is incubated.

TABLE II. ATP Production.

| Incubation mixtures | Yeast only | Yeast + Adenosine, .0375 M | Yeast + Nucleoside, .0375 M | Yeast + Adenosine, .0375 M; nucleoside, .1875 M |
|---|---|---|---|---|
| ATP, $\mu$M/g yeast | 4.2 (4)* | 12.7 (4) | 5.5 (3) | 1.7 (3) |

* No. of experiments.

precipitate 1 is unexplained). Partial hydrolysis of more stable phosphate esters could account for this. Consequently, the materials were analyzed chromatographically for other acid-hydrolyzable phosphate compounds(13). These analyses revealed the presence of 2-phosphoglyceric acid, 3-phosphoglyceric acid, fructose-1-6 diphosphate, and glucose-6-phosphate in each of the precipitates except number IV. The latter contained only inorganic phosphate and glucose-6-phosphate, both of which appeared to be present in larger quantities than in the other precipitates. Glucose-6-phosphate assayed enzymatically substantiated the chromatographic observation. The quantitative data are presented in Table III.

*Discussion.* An inhibitory effect of Aminonucleoside on ATP production from adenosine has been demonstrated in a crude yeast system. In attempting to evaluate the mechanism involved in this inhibition, it must first be noted that formation of ATP in this system is not limited to a single reaction but may proceed as follows(7):

$$\text{Adenosine} + \text{ATP} \xleftrightarrow{\text{adenosine phosphokinase}} \text{AMP} + \text{ADP}$$

$$\text{ATP} + \text{AMP} \xleftrightarrow{\text{myokinase}} 2\ \text{ADP}$$

$$3\ \text{ADP} + 3\ \text{phosphopyruvate} \xrightarrow{\text{pyruvate phosphokinase}} 3\ \text{pyruvate} + 3\ \text{ATP}$$

It is possible then, that inhibition may be at one of several levels. The most likely site, however, would be at the level of adenosine phosphokinase. Direct evidence bearing on this question would require study of the purified enzyme. In this way a competitive type of inhibition might well be demonstrated.

Our data indicate that a significant ATP inhibition occurs in the presence of high concentration of Aminonucleoside. This is confirmed not only by direct measurements of the nucleotides but by associated findings of measurable levels of glucose-6-phosphate in the absence of hexose-di-phosphate and phosphoglyceric acids. This is in sharp contrast to the uninhibited adenosine metabolizing system in which the latter compounds are easily demonstrable. These observations are compatible with formation of glucose-6-phosphate from glycogen, a reaction which does not require ATP. On the other hand, ATP requiring reactions are depressed and this is reflected in the absence of other phosphorylated sugars.

In appraising the relationship between ability of Aminonucleoside to produce the nephrotic syndrome and its inhibitory effect on ATP formation, the following may be pertinent: (1) The earliest lesion observed in animals treated with this nucleoside, which precedes appearance of proteinuria, is the development of glomerular-podocyte damage as noted by Harkin and Recant in electron microscope studies (unpublished) and (2) evidence to date tends to implicate the glomerulus in the etiology of proteinuria in nephrosis (14). It seems reasonable to conclude that the Aminonucleoside may induce proteinuria and initiate the nephrotic syndrome by inter-

TABLE III. Phosphate* Analyses of Ethanol Precipitates.

| Ppt. No. | Chemical analyses: Total $Po_4$ | Inorganic $Po_4$ | Acid-labile $Po_4$ | Enzymatic analyses: ATP | Glucose-6 $Po_4$ |
|---|---|---|---|---|---|
| I | 18.3 | .6 | 4.75 | 3.6 | 11.0 |
| II | 52.45 | 1.41 | 25.3 | 11.5 | 10.8 |
| III | 28.0 | 1.41 | 10.9 | 4.9 | 10.8 |
| IV | 19.0 | 6.50 | 1.87 | 0 | 13.4 |

Ppt. I Yeast only
II " + adenosine .0375 M
III " + aminonucleoside .0375 M
IV " + adenosine .0375 M and aminonucleoside .1875 M

* All data presented as $\mu$M/g yeast.

fering with production of high energy phosphate compounds in key cells within the glomerulus. This hypothesis raises a question concerning enzymatic activity and metabolic requirements of the normally functioning glomerulus. Experiments to explore this question are underway.

*Summary.* Production of acid-labile phosphate by crude brewer's yeast system, in the presence of adenosine and inorganic phosphate, has been demonstrated. This acid-labile phosphate has been identified as adenosine triphosphate. The nucleoside, 6-dimethylaminopurine-3-amino-d-riboside inhibits adenosine triphosphate formation in this system. The possible relationship of this inhibitory action to ability of the nucleoside to produce nephrosis in rats is discussed.

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## Effects of 5-Flurouracil on Human Bronchogenic Carcinoma Grown in Hamster Cheek Pouch.* (24179)

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It has been shown that pyrimidine analogue 5-flurouracil has appreciable tumor-inhibitory activity against a variety of transplantable rat and mouse tumors(1). The work described here is a preliminary study of the efficacy of this drug on heterologously grown human bronchogenic carcinoma transplanted in the hamster cheek pouch.

*Materials and methods.* The tumor was the Skiff Bronchogenic Carcinoma obtained from Sloan-Kettering Institute for Cancer Research, N.Y. City. The host animal used was the golden hamster (*Mesocricetus auratus*), and site of tumor growth was the left cheek pouch. Experimental animals 40 to 80 g were obtained from National Pet Shops, St. Louis, Mo. Animals of both sexes were used. The tumor transplant was introduced into the cheek pouch under light ether anesthesia with a No. 14 trocar, by the method of Lutz, *et al.*(2) as modified by Toolan(3), Patterson, *et al.*(4) and Palm, *et al.* (unpublished). Microscopic examination of a hematoxylin eosin stained section at time of transplantation revealed that the tumor was composed of fairly uniform cells of moderate size with hyperchromic nuclei and fairly large amounts of deeply staining cytoplasm. The cells tended to be spindle-shaped and elongated in many areas. Only rare giant nuclei were present and multi-nucleated forms were not seen. A bronchogenic tumor in man with these morphologic characteristics would usually be classified as an undifferentiated carcinoma of the "large cell" type. The tumor specimen used for transplantation was harvested from the cheek pouch of a maintenance animal, in which it had grown for 14

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TABLE I. Treatment with 25-30 mg/kg/Day of 5-Flurouracil for 7 Days.

| Control | | Treated | |
|---|---|---|---|
| Wt of | | Wt of | |
| Host (g) | Tumor (mg) | Host (g) | Tumor (mg) |
| 59 | 789 | 56 | 709 |
| 66 | 375 | 82 | 1054 |
| 64 | 182 | 57 | 338 |
| 64 | 178 | 54 | 928 |
| 49 | 260 | 65 | 363 |
| 63 | 466 | 56 | 383 |
| | | 65 | 179 |
| 61 ± 1.2 | 375 ± 95 | 62 ± 6.1 | 565 ± 126* |

* Mean ± stand. error of mean.

days. The animals were placed under light ether anesthesia 5 days after implantation of tumor. Animals in which tumors did not take were discarded and tumor bearing animals were divided randomly into treated and control groups. All animals were given cortisone: 50 mg/kg, injected subcutaneously into nape of neck, every 4 days throughout experiment (Cortone Acetate, Merck, Sharp and Dohme). 5-Flurouracil treatment was begun on fifth day after transplantation (animals had received 2 cortisone injections), and was continued for 7 days or until the drug proved to be too toxic (as indicated by marked weight loss). 5-Flurouracil in crystalline form was obtained from Dr. Charles Heidelberger (Univ. of Wisconsin Medical School). 5-Flurouracil was dissolved at concentration of 4 mg/ml of distilled water and stored at 5°C. Control animals were given 0.45% saline solution prepared by dilution of physiological saline with distilled water. 5-Flurouracil and saline control solutions were administered intraperitoneally. At termination of each experiment, animals were weighed and autopsied. Tumors of treated and control animals were weighed. In addition, gross toxic effects on various organisms were noted, and histologic sections of treated and control tumors were made. The possible anti-tumor action of 5-flurouracil was tested at 2 dosage levels: 25-30 mg/kg/day and 50-60 mg/kg/day. The lower level was the optimal dosage used by other workers with homologously grown rat and mouse tumors(1).

*Results.* Data in Table I show that with dosage level of 25-30 mg/kg/day of 5-flurouracil for 7 days there was no inhibition of growth of the Skiff Human Bronchogenic Carcinoma grown in the hamster cheek pouch. The average weight of treated tumors at end of 7 days was 565 mg, the control tumors weighed 375 mg; the difference of these values was not statistically different. There was also no significant difference in weights of treated and control animals, indicating that the drug had no toxic effects by this criterion. Gross examination of spleens, livers and gastrointestinal tracts of treated animals revealed no abnormalities. Microscopic examination of treated and control tumors revealed no difference in histological appearance.

Data in Table II show that with higher dosage level of 5-flurouracil, 50-60 mg/kg/day, for 3 days there was marked inhibition of the heterologously grown human bronchogenic carcinoma. The average weight of treated tumors was 74.5 mg; that of control tumors was 358.0 mg. The weights of necrotic tumors were not included in the final tabulation nor were the weights of tumors in animals who died prior to termination of experiment.

There was also a marked and significant loss of weight in the treated group of animals indicating extremely toxic effects of the drug at dose given. The data in Table III show that treated animals dropped from average weight of 60 g, on day that treatment was

TABLE II. Treatment with 50-60 mg/kg/Day of 5-Flurouracil for 3 Days.

| Control | | Treated | |
|---|---|---|---|
| Wt of | | Wt of | |
| Host (g) | Tumor (mg) | Host (g) | Tumor (mg) |
| 63 | 350 | 52 | 30 |
| 80 | 81 | 50 | 29 |
| 68 | 91 | 64 | 180 |
| 60 | 114 | 40 | 59 |
| 48 | 457 | 55 | 59 (necrotic) |
| 52 | 742 | 38 | 28 ( " ) |
| 52 | 666 | 39* | |
| 58 | Necrotic | 48* | |
| 54 | " | 44* | |
| 68 | " | 50* | |
| | | 49* | |
| 60 ± 3.3 | 358 ± 104.5 | 46.9 ± 2.2 | 74.5 ± 35.0 † |

* Died after 3 days of treatment.
† Mean ± stand. error of mean.
Weights of necrotic tumors not averaged.

TABLE III. Summary of Effects of 5-Flurouracil on Host Animals.

| Experiment | Group | Wt of host (g) Prior to treatment | Wt of host (g) After treatment | Animals surviving/animals treated |
|---|---|---|---|---|
| Exp. 1—25-30 mg/kg/day of 5-flurouracil for 7 days | Control | 61.9 ± 4.3 | 61.0 ± 2.8 | 6/6 |
| | Treated | 64.9 ± 4.2 | 62.0 ± 3.7 | 7/7 |
| Exp. 2—50-60 mg/kg/day of 5-flurouracil for 3 days | Control | 59.3 ± 4.8 | 60.3 ± 3.3 | 10/10 |
| | Treated | 60.0 ± 2.9 | 46.9 ± 2.2 | 5/11 |

begun, to average weight of 46.9 g after 3 days of treatment with drug at higher dosage level. In the control group in the 3-day period there was no decrease of average weight. Five of 11 animals died after 3 days of treatment. Further indications of the toxicity of the drug were that 50% of treated animals showed infection and necroses of the stomach, small and large intestine and pallor of liver on gross examination. The average weight of spleens of treated animals was 45.8 mg (= .098% body weight) that of controls 102 mg (= .168% body weight). In addition, all treated animals showed a marked loss of fur, not noted in the control group.

Histological examination of the treated tumor revealed no morphological difference from control tumors.

*Discussion.* It is interesting to note that 5-flurouracil when administered at dosage level of 25-30 mg/kg/day for 7 days showed no incidence of cytotoxic effect on either host or tumor, while double this dose for 3 days had a very drastic effect on both. These effects are summarized in Table III. Future work with this drug will be aimed at determining whether there is any dosage level between the apparently ineffective 25-30 mg/kg and the highly toxic 30-60 mg/kg at which a specific toxic effect on the tumor can be demonstrated.

*Summary.* 5-flurouracil at dosage level of 50-60 mg/kg/day produced a marked tumor inhibitory effect on human bronchogenic carcinoma grown in the hamster cheek pouch but was very toxic to the host. A dose of 25-30 mg/kg/day had no apparent effect on either tumor or host.

We are indebted to Dr. Valentina Suntzeff of the Wernse Cancer Research Laboratory, Washington University, St. Louis, Mo., for the tumor maintained in her laboratory, and to Dr. Joy Palm of Sloan-Kettering Institute for Cancer Research, N. Y. City, who made the tumor available to Wernse Laboratory. We also thank Dr. Morris Friedkin of Washington Univ. Dept. of Pharmacology for his technical assistance, and Dr. Robert O'Neal, Dept. of Pathology, and Dr. Suntzeff for help in histologic studies.

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# Response of Arterial Wall to Intramural Cholesterol.*† (24180)

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Winternitz *et al.*(1) have suggested that hemorrhage from the vasa vasorum is the first step in pathogenesis of atherosclerosis. Cholesterol and cholesterol esters left behind after resorption of more soluble constituents of blood, were thought to be capable of inciting intimal proliferative changes. Wartman and Laipply(2) injected homologous whole blood into wall of femoral artery of dogs to establish the role of intramural hemorrhages in atherogenesis. Medial scarring was observed, but intimal changes were not found. Christianson(3) produced intimal proliferative changes in the dog by intramural injection of cholesterol dissolved in human fat. Interpretation of these findings is difficult, however, because of the complexity of the injected material.

*Materials and method.* White Leghorn cockerels of 1 to 1.5 kg were anesthetized with intravenous sodium pentobarbital (30 mg/kg). The cockerels were then intubated with a soft rubber catheter as endotracheal tube. Artificial respiration was maintained by means of pump with alternating positive pressure. An incision then was made between third and fourth ribs on left side. The heart, aortic arch and left brachiocephalic artery were exposed by retracting the ribs. This resulted in an orifice through which the brachiocephalic artery could easily be pulled up to insert a 20 gauge needle. The material injected was 0.2 ml of a 20% suspension of crystalline cholesterol in 0.9% saline solution. All injections were made directly into the adventitia or media of left brachiocephalic artery. Four groups of 10 birds each were handled in this manner. Diets of individual groups were: 1) growing mash only; 2) growing mash plus 8% lard; 3) growing mash plus 8% corn oil; 4) growing mash plus 4% lard, 4% corn oil and 1% cholesterol. Two birds from each group were killed at 2 week intervals over a 10 week period and the right and left brachiocephalic arteries were removed for study. The right brachiocephalic artery served as control for spontaneous appearance of atherosclerosis. In addition, a group of 10 birds were fed only growing mash and were injected with 0.2 ml of 0.9% saline solution without cholesterol to determine the effect of trauma by needling. The arteries were embedded in paraffin and studied in step sections at 200 $\mu$ intervals. Histologic staining technics used were: hematoxylin and eosin, periodic acid-Schiff (P.A.S.), Verhoeff's elastic tissue stain, and phosphotungstic acid hematoxylin (P.T.A.H.).

*Results.* A foreign-body reaction to cholesterol was noted in adventitia or media at site of injection. This consisted of foreign-body giant cells, cholesterol slits, and increased amounts of connective tissue. Most injections were in the adventitia.

The most striking finding, however, was a focal proliferation of collagenous connective tissue in the intima (Fig. 1). Definite proliferative changes were found as early as 2 weeks after injection of cholesterol, but reached full development in a month. This reaction was present although often the media was anatomically intact. As a result of localized increase in connective tissue, the lumen was partially occluded and on occasion was almost completely obliterated. The plaques were P.A.S. negative and elastic tissue was not present. In the P.T.A.H. preparations, the reaction was that of collagenous connective tissue. The connective tissue plaques were often at a distance from the foreign-body reaction. Endothelial cells occasionally were increased in number. In the

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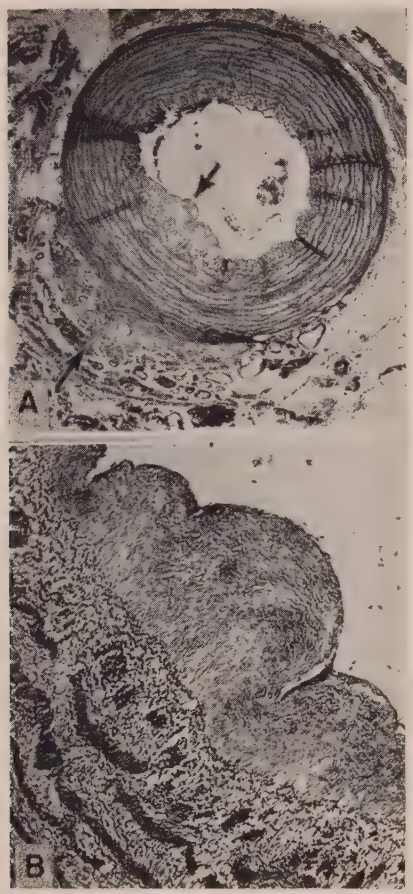


FIG. 1. (A) Cross-section of avian artery showing response to cholesterol in adventitia. A foreign-body reaction (lower arrow) is seen in the perivascular space, and an intimal plaque protrudes into the lumen (upper arrow). Note that the media is intact. Hematoxylin and eosin technic, × 15. (B) Higher power view of proliferated connective tissue in the intimal plaque. Hematoxylin and eosin technic, × 110.

P.T.A.H. preparations, the reaction was that of collagenous connective tissue. The connective tissue plaques were often at a distance from the foreign-body reaction. Endothelial cells occasionally were increased in number. Spontaneous atherosclerosis was not found under the experimental conditions. The saline injected controls revealed only a needle tract. Connective tissue and intimal proliferation were not present.

Intimal plaques occurred in 75% of birds injected with cholesterol. This proportion was similar in each of the 4 dietary groups. The development of lesions, therefore, bore no relation to degree of saturation of ingested fat or to enrichment of diet with cholesterol. The lesions are therefore the result of local deposition of cholesterol and are not affected by the diet.

*Summary.* A technic is described for study of response of avian arteries to intramural injection of cholesterol. This material, when injected into the wall of the artery, causes a foreign body reaction at site of injection. In addition, a localized proliferation of collagenous connective tissue is found in the intima. These intimal plaques resemble some forms of human atherosclerosis. The development of the plaques is not affected by adding cholesterol or various fats to the diet. These findings suggest that the lesions are the result of the local metabolic alteration.

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# Histological Effect of Chlorpromazine on Nissl Substance and Golgi Apparatus of Cortical Neurons. (24181)

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Holtet(1) reported a marked depletion of Nissl substance, especially in the large pyramidal cells in the cerebral cortex of the albino rat, following administration of chlorpromazine in doses ranging 20 to 60 mg/kg/day for 3 days. He presented several photomicrographs of large pyramidal cells, stained with Einarson's gallocyanin-chromalum method, which were completely devoid of Nissl substance and showed marked swelling of neurons. This he believed was a reaction to injury and that chlorpromazine in some way interfered with metabolism of Nissl bodies. This work stimulated us to attempt to confirm these observations and to study other possible abnormalities in cytological structure of the cortical neurons.

*Materials and methods.* Twenty-eight C57 mice received 15 mg/kg/day chlorpromazine* subcutaneously for 28 days. Fourteen mice received placebo injections for same period. On 29th day the mice were decapitated, the cranial vault emptied and the contents placed in appropriate fixatives. In addition 3 adult Osborn-Mendel rats were injected interperitoneally with 25 mg/kg of chlorpromazine 3 times a day for 3 days. Three control rats were injected with an equivalent amount of placebo. On the 4th day the rats were sacrificed in the same manner as mice. Mouse tissue was stained by Einarson's gallocyanin-chromalum technic(2) and also with toluidine blue. Rat brain sections were stained for Nissl bodies utilizing the gallocyanin-chromalum method and the methyl-green pyronin Y method of Long and Taylor(3). Golgi apparatus in both mouse and rat tissue was stained by silver techniques of Kruszynski(4) and the method of Elftman(5), followed by gold toning.

*Results.* Following injection of chlorpromazine, the animals became quite lethargic, indifferent to their surroundings and appeared to be sleeping, however they were easily aroused by handling. Despite this dramatic change in behavior there were no detectable differences in Nissl structure of the experimental animals when compared to their controls. The sections stained by Einarson's gallocyanin-chromalum method, methyl-green pyronin Y method, and the toluidin blue technic revealed essentially normal dense clumps of chromophilic substance in the cytoplasm of pyramidal cells of the cortex. As a control on staining procedures, several sections were washed in 1% ribonuclease for one hour at room temperature. In these sections the pyramidal cells were completely devoid of stainable Nissl substance.

The Golgi apparatus in cortical cells of experimental animals appeared essentially normal, presenting a fine perinuclear reticulum, but in some cells the network was broken up somewhat into fine granules. We failed to demonstrate any consistent changes in Golgi apparatus of the pyramidal cells of either the experimental mice or rats.

*Discussion.* In dogs chlorpromazine is concentrated primarily in the brain(6). Using $S^{35}$ labeled chlorpromazine, Christensen and Wase(7) further showed a marked uptake by the brain in 30 minutes, maintained for 2 days with maximal excretion in urine occurring in 4 to 24 hours. The exact site and mechanism of action within the brain, however, is still in doubt. Lehmann(8) contends that the site of action is in the ventral region of the thalamus, in the brain stem, in the subthalamic nucleus, red nucleus, substantia nigra, hypothalamus, and the pontile tegmentum, while Himwich(9) believes the posterior hypothalamus and the midbrain reticular gray are primarily involved.

After completion of this work, however, we learned by personal communication with Dr. Larus Einarson, in whose laboratory Holtet's

* Generously supplied by Smith, Kline, and French Laboratories as Thorazine.

work was done, that upon reevaluation of their data they felt that these results were due, at least in part, to traumatization to the animals during injections of the drug. This leaves Grenell's(10) observation that the nucleic acids are reduced in chlorpromazine treated rats unconfirmed. It is, of course, possible that these biochemical alterations in nucleic acids are insufficient to be detected by the usual histochemical technics.

*Summary.* In mice receiving 15 mg/kg/day of chlorpromazine for 28 days and in rats receiving 25 mg/kg of the drug 3 times a day for 3 days, no morphological alterations in the Nissl and Golgi bodies of the cells of the cerebral cortex were noted.

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## Observations on Toxins of Poisonous Fishes.* (24182)

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Certain fishes of the tropical Pacific, including fish as diverse as puffers (*Tetraodon*) and snappers (*Lutjanus*), have long been reported to be highly toxic when eaten. Hiyama(1), by feeding tests, and Halstead and Bunker(2), by intraperitoneal injections into mice of aqueous extracts of fish viscera and flesh, have confirmed these reports. This toxin is not the result of bacterial decomposition, but occurs in the flesh of fresh fish. These toxins may vary among the different species(3). The object of these tests was to explore the nature of the toxin sufficiently to permit planning of future studies on poisoning characterized by Halstead(3) as *Ciguatera*. Fish producing this syndrome are reported by islanders to be highly toxic in certain restricted areas, but elsewhere are excellent as food. To avoid any confusion of toxins that could be different, only body muscles of *Lutjanus bohar* Forskal from Palmyra were used.‡ About 20 fish were tested ranging 14 to over 24 inches long. Only fish longer than 16 inches produced the syndrome characteristic of *Ciguatera* poisoning.

*Results.* The first phase was to find an animal that would respond consistently with symptoms paralleling those found in man. Cats so responded, as reported by others, but were not readily available. A variety of other vertebrates was then tested, but none except the imported mongoose (*Herpestes javanicus auropunctatus*) responded to dosages, in terms of body weight, that caused paralysis and death in cats. The symptoms of the mongoose, like those of the cat, included loss of certain reflexes, for example, failure to withdraw the foot when touched lightly. A progressively severe loss of coordination followed in first the back and then the forelegs which, in acute cases, would prevent the mongoose from standing or righting itself after falling. Death, preceded by coma and

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‡ We wish to thank the U. S. Fish and Wildlife Service for catching the fish which were frozen immediately after capture.

Cheyne-Stokes breathing, would occur within 36 to 48 hours, if fish of sufficient toxicity was fed. Unlike cats and man, the mongoose never vomited, thereby permitting a more accurate assessment of the amount of fish eaten.

In tests reported below the mongoose was used exclusively; no mongoose, after once showing symptoms, was used again. Mongooses were fed raw fish to determine approximate lethal dose. No fish was found to be lethal under 5% body weight, and no fish was used that did not cause death at 15% body weight. When fluid extracts of the fish were used, they were concentrated and evaporated on Gaines dog food for feeding.

Feeding tests confirmed that toxin in fish is not noticeably reduced by drying the minced muscle at 102°-108°C for 24 hours, or by freezing the whole fish for periods up to 6 months.

Solubilities of the toxin were explored. The implication of test reported by Halstead(2,4) is that the toxin is soluble in distilled water. Ground muscle, thrice extracted with its own weight of distilled water for 24 hours, showed no loss in toxicity, but the extract, concentrated on dog food, caused no reaction when fed.

When dehydrated ground flesh was extracted with chloroform, diethyl ether, or hot absolute alcohol, neither the extract nor residue fed at twice the lethal dosage produced obvious reactions. When dried meat was extracted with 90% ethanol, all of the toxin appeared in the extract; 70% ethanol appeared to be less efficient, and absolute alcohol extracted almost no toxin. The 90% ethanol extract decreased in toxicity when it was evaporated to dryness without dog food.

When secondary extraction of the 90% ethanol solution was made with repurified ether, dioxane, chloroform or petrol ether, no observable toxicity was left in either fraction. However, under an atmosphere of commercial nitrogen (about 3% oxygen) toxicity of extracts was diminished but not lost. When the alcoholic solution was evaporated to dryness under nitrogen, then extracted with petrol ether and the undissolved residue redissolved in 90% ethanol, both fractions gave toxic symptoms. When the experiment was repeated with chloroform or diethyl ether, toxicity was observed only in the chloroform and diethyl ether solutions.

The reliability of Halstead's technics(2,4) was also investigated. Halstead homogenized portions of fish, *i.e.* muscles, viscera, etc., in water, centrifuged the slurry for 25 minutes at 2,000 rpm, and injected 1 ml of supernatant fluid intraperitoneally in 15 to 25 g mice.

Halstead recognized 3 types of mouse reactions to poisonous fish: *weakly positive,* including lacrimation, diarrhea, and ruffling of hair, but the animal recovered; *moderately positive,* including same symptoms, but the animal died within 36 hours; and *strongly positive,* where test animal died within one hour.

Mice showed no symptoms when fed toxic fish even at twice the dose in percentage of body weight that was lethal to a mongoose. An extract was prepared according to the method of Halstead and Bunker(2,4) of a lethally toxic fish and of a non-toxic tuna; these extracts were injected into mice weighing from 20 to 25 g. The 2 mice receiving the tuna injection showed no reaction, but the 2 receiving the lethal fish displayed what Halstead called a "weakly positive" reaction, with slight lacrimation, diarrhea, and ruffled fur.

The extract prepared by this technic was not a solution but a milky suspension. Since mice injected with the most toxic *Lutjanus* available did not die, a fish classified by Halstead as having a different type of toxin was used to determine whether the toxin was in solution or suspension. Liver and gonads of the puffer [*Arothron hispidus* (L.)] were prepared by Halstead's technic; after centrifugation the extract was divided into 2 portions. One portion was filtered twice through filtered paper and finally through a Millipore glass filter. Two mice, weighing between 20 and 25 g, were each injected intraperitoneally with one ml of this solution, while the unfiltered extract was injected into 2 similar mice. The 2 mice receiving the *unfiltered* extract

died but the ones injected with the filtered extract showed no symptoms.

*Summary.* 1. Not all animals are sensitive to the *Ciguatera*-type toxin. 2. This toxin is not water soluble, but is soluble in 90% ethanol and certain other solvents. 3. The toxin may be detoxified when concentrated in normal atmospheric oxygen, but remains toxic under nitrogen. 4. The test previously used to assess this toxicity is not sufficiently sensitive and depends upon the suspended matter in the extract.

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## Ability of Human Plasma to Lyse Homologous Erythrocytes Pretreated with Fatty Acid. (24183)

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Addition of washed mammalian erythrocytes to fatty acid solutions results in hemolysis, the rate dependent upon fatty acid and erythrocyte concentration and upon pH and temperature(1). Addition of homologous plasma or plasma protein fractions to fatty acid *prior* to insertion of washed erythrocytes has long been known to retard or inhibit hemolysis(2-4). This inhibiting effect has been attributed to binding of fatty acid by plasma constituents, either in solution or at surface of red blood cell(5). To date, we are unaware of any data indicating action of homologous plasma or plasma protein fractions *after* erythrocyte contact with fatty acid. This report describes ability of human plasma to lyse homologous erythrocytes pretreated with fatty acid.

*Materials and methods.* All fatty acid solutions were prepared in sodium phosphate buffered saline, ionic strength 0.15, pH 7.2. Normal human erythrocytes of all major blood groups were tested. Erythrocytes were washed 3 times in buffered isotonic saline and resuspended in a concentration such that 0.5 ml added to 2.5 ml of fatty acid solution resulted in 5 x $10^5$ cells/cu. mm. Human plasma protein fractions were prepared by dialysis of plasma against ammonium sulfate at 4°C. The globulin-rich fractions were precipitated at 50% ammonium sulfate saturation and the residual albumin-rich fractions at 100% saturation. All precipitates were washed with appropriate ammonium sulfate solutions, resuspended in buffered isotonic saline, and dialyzed at 4°C against isotonic saline until free of ammonium ion (verified with Nessler's reagent). The final volume of plasma fractions was adjusted with buffered isotonic saline (pH 7.4) to equal that of original plasma volume. Human erythrocytes were added to varying concentrations of sodium oleate at 25°C for 3 minutes. The red blood cells were then spun at 3000 rpm, 0°C for 3 minutes and washed 5 times with 5 ml aliquots of iced buffered saline. Following the last washing, the packed red blood cells were rewarmed to 25°C and 1 ml of homologous plasma or homologous plasma protein fraction added with gentle agitation. After 2 minutes at 25°C, erythrocyte suspensions were spun at 3000 rpm, 0°C for 3 minutes and resulting hemolysis determined with the Klett-Summerson photoelectric colorimeter (filter 550 m$\mu$).

*Results.* Over one hundred tests were performed with varying concentrations of fatty acid. Typical results are given in Table I.

TABLE I. Ability of Human Plasma and Plasma Fractions to Lyse Homologous Erythrocytes Pretreated with Fatty Acid.

| Solution added to packed human erythrocytes washed 5 times after pretreatment with oleate (1.0 ml) | % hemolysis 5 min. after addition of plasma or plasma fractions* Pretreatment oleate conc.: $1 \times 10^{-4}$ M | $.3 \times 10^{-4}$ M |
|---|---|---|
| Isotonic buffered saline (control) | 15 | 0 |
| Homologous plasma | 48 | 0 |
| Dialyzed homologous plasma | 50 | 0 |
| Homologous plasma diluted 1:16 with isotonic saline | 48 | 0 |
| Homologous albumin fraction | 100 | 100 |
| Homologous globulin " | 100 | 100 |
| Homologous albumin + globulin fractions, mixed 1:1 | 100 | 100 |
| Homologous plasma saturated with sodium oleate† | 17 | 0 |
| Homologous albumin fraction saturated with sodium oleate† | 18 | 0 |
| Homologous globulin fraction saturated with sodium oleate† | 17 | 0 |

* Mean of 4 determinations on a single batch of plasma and erythrocytes. Per cent hemolysis determined photometrically on supernates prepared by centrifugation at 3000 r.p.m., 0°C for 3 min.

† Oleate binding saturation considered attained with maximum quantity of added oleate failing to lyse washed human erythrocytes at 37°C in 3 hr.

The findings indicate:

A) Human plasma or albumin or globulin fractions hemolyze washed homologous erythrocytes pretreated with sodium oleate. Similar results were obtained with heparinized, oxalated, or citrated plasma, and with serum.

B) Lytic activity of homologous plasma albumin and globulin fractions for oleate pretreated erythrocytes is more intense than that of whole plasma. This cannot be ascribed to dialysis or to protein losses in preparing albumin and globulin fractions since dialysis or 16 fold dilutions of whole plasma does not alter the results. Rather the findings suggest the presence of a lytic inhibitor in whole plasma which can be removed or inactivated by ammonium sulfate fractionation. Studies now in progress suggest that this inhibitor is plasma unesterified fatty acid.

C) Saturation of oleate binding capacity of human plasma or plasma protein fractions by pretreatment with exogenous oleate inhibits the lytic activity of plasma for the oleate pretreated erythrocytes. These findings suggest that human plasma and plasma albumin or globulin fractions lyse homologous erythrocytes by combining with oleate molecules fixed to the surface of the red blood cell. Whether plasma acts by "tearing" the oleate molecules from the cell membrane or by forming a new surface complex is as yet undetermined. Failure to prevent the lytic reaction by repeated washings of the oleate pretreated erythrocytes indicates the firmness of the oleate-erythrocyte bond.

Concentration of sodium oleate utilized to pretreat human erythrocytes was an important determinant of lysis following exposure to homologous plasma. Human erythrocytes exposed to oleate at concentrations which, after repeated washings, fail to cause hemolysis faster than that of untreated red blood cells, were not lysed by homologous plasma or plasma fractions. Human plasma and plasma albumin and globulin fractions thus appear to act upon homologous erythrocytes pretreated with oleate by accelerating oleate hemolysis. This does not necessarily imply that the accelerated lysis occurs *via* the same intermediate steps as the control oleate lysis.

The present findings are not restricted to oleate or to human erythrocytes. The other fatty acids tested, stearate and palmitate, yielded hemolytic reactions with homologous human plasma qualitatively similar to those of oleate. Moreover, plasma of rats, guinea pigs, sheep, and rabbits were also found capable of accelerating lysis of homologous erythrocytes pretreated with fatty acid.

*Summary.* Addition of human plasma to washed homologous erythrocytes pretreated with sodium oleate results in hemolysis. Whole plasma is a less efficient lytic agent than is the homologous albumin or globulin moieties prepared by ammonium sulfate fractionation. Saturation of fatty acid binding capacity of plasma or plasma protein fractions, by addition of exogenous oleate inhibits lytic activity. Hemolysis appears related to acceleration of lytic activity of oleate molecules fixed to the surface of the red blood cell following binding by plasma constituents. Similar results were obtained with other fatty

acids and with erythrocyte-plasma systems from other mammalian species.

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## Deiodination of Thyroxine and Other Iodinated Compounds During Desalting by Electrodialysis.* (24184)

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Paper chromatography of thyroxine and its derivatives in a Ringer solution used for tissue metabolism studies is often unsatisfactory because the salts lead to training of the spots. Since desalting of solutions of ordinary amino acid can easily be accomplished using the electrolytic desalter of Consden, Gordon and Martin(1), an apparatus based on their design (Research Equipment Co.) was tried. The present study of rapid deiodination confirms and considerably extends the alterations in some iodinated compounds reported by Smith *et al.*(2) and by Jepson and Smith(3).

*Methods.* The desalter achieves results by electrolytic reduction of cations which are amalgamated at the cathode and passage of anions through a cellophane membrane into a flushing electrolyte solution. Conditions were chosen to remove completely the $Na^+$ and $Cl^-$ from an isotonic NaCl solution, starting with 10 volts D.C. and 5-12 amps. The operation was finished in 10-15 minutes, as indicated by decrease in current to 0.2 amp. About 1 to 2 mg of iodinated substance† were dissolved in 0.05 ml N NaOH and diluted to 4 ml with 0.9% NaCl. Of this solution, 3.5 ml were treated in the chamber of the desalter. Aliquots representing before and after deionization were subjected to ascending chromatography, using n-butanol, ammonium hydroxide, water (250:72:178); n-butanol, glacial acetic acid, water (78:5:17); or methanol and 0.2 M ammonium acetate (100:250). The amino acids were revealed with ninhydrin(4, p. 88), phenolic substances with the Pauly reagent(4, p. 253) and iodine-containing compounds with ceric sulfate-arsenious acid(5).

*Results.* Thyronine, thyrosine and phenylalanine solutions all were desalted without loss of amino acids. The $R_F$ values are shown in Table I. In contrast, mono- and diiodotyrosine were rapidly deiodinated to tyrosine. 3,3′-Diiodothyronine, 3,5,3′-triiodothyronine and thyroxine were deiodinated to thyronine, although in the last case, some free iodide remained in the cathode compartment. The iodine was also completely removed from 3,5-diiodothyronine, but some additional change was produced, since the product was clearly not thyronine. Further identification has not been possible, beyond demonstrating (Table I) that the material is a single phenolic amino acid (Pauly and ninhydrin-positive) not containing iodine. From the intensity of the ninhydrin and Pauly stains, it is probable that there was no loss of amino acid itself.

Table II shows the results of desalting car-

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TABLE I. Desalting of Derivatives of Thyroxine.

| Original compound | $R_F$ before desalting* 1 | 2 | 3 | $R_F$ after desalting* 1 | 2 | 3 | Resulting compound |
|---|---|---|---|---|---|---|---|
| NaI | | .11 | .78 | | | | |
| Phenylalanine | .24 | .28 | | .24 | .27 | | |
| Tyrosine | .05 | .14 | | .05 | .14 | | |
| Thyronine | .34 | .44 | | .34 | .43 | | |
| 3-Monoiodotyrosine | | .28 | | | .14 | | Tyrosine |
| 3,5-Diiodotyrosine | | .41 | | | .13 | | " |
| 3,3′-Diiodothyronine | .27 | .62 | | .28 | .43 | | Thyronine |
| 3,5,3′-Triiodothyronine | .46 | .63 | | .35 | .41 | | " |
| Thyroxine | .29 | .67 | .16 | .34 | .41 | .78 | " |
| 3,5-Diiodothyronine | .25 | .34 | | .18 | .23 | | Unknown |

* Solvent systems: 1. Butanol-ammonia-water. 2. Butanol-acetic acid-water. 3. Methanol-ammonium acetate.

ried out on several thyroxine derivatives having fatty acid side chains in place of the alanine. Again, some deiodination was apparent, but specific identification of the products was not possible because information concerning the non-iodinated compounds was lacking. 3,5-Diiodothyroacetic and 3,5,3′-triiodothyroacetic acids were completely deiodinated to the same material, presumably 4-(4′-hydroxyphenoxy)-phenylacetic acid. The tetraiodothyroacetic acid was partially recovered unaltered. Since iodide was also encountered, some of the tetraiodothyroacetic must have been deiodinated to the iodine-free compound just mentioned, although not in large enough quantity to give a Pauly stain.

Tetraiodothyropropionic acid was only partly deiodinated, but 3,5,3′-triiodothyropropionic was completely stripped of iodine. With all 5 organic acid compounds, deiodination was sufficiently slow that traces of inorganic iodide could be detected in the solution with the extremely sensitive iodide test.

Tetraiodothyroacrylic acid proved to be far more stable than any other material, resisting alteration completely. In contrast, 3,5-diiodothyroacrylic and tetraiodothyroformic acids not only were completely deiodinated, but were both sufficiently altered that no Pauly reaction was obtained. This suggests that there may have been reduction of the hydroxyl or more drastic alteration of the ring structure.

Table III shows the effects of desalting sev-

TABLE II. Desalting of Fatty Acid Analogues of Thyroxine.

| Original compound | $R_F$ before desalting* 1 | 2 | 3 | $R_F$ after desalting* 1 | 2 | 3 | Resulting compound |
|---|---|---|---|---|---|---|---|
| 3,5-Diiodothyroacetic | .33 | .88 | .61 | .33 | .88 | .44 | Non-iodinated† |
| | | | | | .11 | .77 | NaI |
| 3,5,3′-Triiodothyroacetic | .50 | .88 | .53 | .33 | .89 | .44 | Non-iodinated† |
| | | | | | .11 | .79 | NaI |
| Tetraiodothyroacetic | .47 | .85 | .45 | .41 | .86 | .44 | Tetraiodothyroacetic |
| | | | | | .11 | .79 | NaI |
| 3,5,3′-Triiodothyropropionic | .57 | .88 | .53 | .48 | .87 | .53 | Non-iodinated† |
| | | | | | .11 | .83 | NaI |
| Tetraiodothyropropionic | .43 | .85 | .32 | .41 | .90 | | Non-iodinated† |
| | | | | | .11 | .76 | NaI |
| 3,5-Diiodothyroacrylic | .67 | | | — | | | None detectable |
| Tetraiodothyroacrylic | .46 | | | .46 | | | Tetraiodothyroacrylic |
| Tetraiodothyroformic | .53 | | | — | | | None detectable |

* Same solvent systems as Table I.

† The same non-iodinated, Pauly-positive compound in all 3 instances; probably desiodothyroacetic acid.

TABLE III. Desalting of Various Iodinated Compounds.

| Original compound | $R_F$ before desalting* 1 | 2 | 3 | $R_F$ after desalting* 1 | 2 | 3 | Resulting compound |
|---|---|---|---|---|---|---|---|
| O-Methyl thyroxine | .53 | .61 | | .42 | .48 | | Deiodination |
| Thio-ether analogue of thyroxine | .35 | .75 | | .33 | .71 | | " |
| Thyroxamine | .85 | .79 | | .90 | .76 | | " |
| 3′, 5′-Dichloro-3,5-diiodothyronine | .30 | .73 | | .18 | .48 | | |
| 3′, 5′-Dibromo-3,5-diiodothyronine | .19 | .79 | | .25 | .38 | | Deiodination (thyronine?) |
| 2,6-Diiodophenoxyacetic | | | | | .10 | | NaI |
| 3,5- " | | | | | .10 | | " |
| Tetraiodophenolphthalein | .56 | .94 | | .55 | .94 | | No change |

* Same solvent systems as Table I.

eral other iodinated compounds, some derivatives of thyroxine and others quite different in structure. Since none of the deiodinated products is available, it is only possible to consider that loss of iodine and change of $R_F$ demonstrate at least deiodination. Other alterations may be produced, similar to those found in 3,5-diiodothyronine (Table I).

O-Methyl thyroxine, the thio-ether analogue of thyroxine and thyroxamine were all deiodinated, the last much more slowly. Since 3′,5′-dichloro- and 3′,5′dibromo-3,5-diiodothyronines were deiodinated, it is probable that dehalogenation was complete, but the $R_F$'s obtained after treatment are not truly the $R_F$ of thyronine. Tetraiodophenolphthalein was completely stable. Two iodophenoxyacetic acids did not react with the ceric-arsenious acid reagent before treatment, but did show the presence of some inorganic iodide afterwards.

*Conclusions.* 1) Use of electrolytic desalter to remove inorganic ions from solution, results in removal of iodine from aromatic linkage, including thyroxine, triiodothyronine, diiodotyrosine and several of the fatty acid side-chain analogues of thyroxine. 2) This prevents use of apparatus for preparation of salt-free solutions in thyroid studies. The procedure might be useful in preparation of various dehalogenated derivatives where the parent compounds are readily available.

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## Lactic Dehydrogenase Production in Tissue Culture of Normal, Transformed, and Malignant Human Cell Lines.*† (24185)

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The lactic dehydrogenase activity (LD) of serous effusions containing and/or bathing proliferating malignant neoplastic cells has been found greater than LD activity of blood plasma from which the serous effusion is derived. It was postulated that the greater enzyme activity might be due to its accumulation in serous fluid as a result of either multi-

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plication or disintegration of malignant cells (1). Since many strains of malignant cells are well established in tissue culture, it seemed that information relative to the contribution of LD activity to the fluid bathing them might be gained by a study of their enzymatic behavior in tissue culture. In addition, these same methods could be used to characterize enzymatic behavior of established cell lines known to have been isolated from carcinomas as well as those which were isolated from normal tissue, but which during their serial passage in tissue culture had changed their characteristics(2). Normal cell lines derived from first generation amnion cells or from recently isolated fibroblastic cell lines were used for comparison.

*Materials and methods. Cell lines.* The following human cell lines have been used: from known malignant sources—HEP #1, HEP #2(3), Skiff(4), and J-111(5); from normal tissues which changed in tissue culture —Chang's liver and conjunctiva(6) and FL amnion(7); from normal sources—normal amnions prepared and planted in tissue culture according to standard methods(8) and human fibroblasts, derived either from adult or embryonic sources, which had had less than 7 trypsinizations and which morphologically and chromosomally appeared unchanged. All cell lines were grown in Eagle's synthetic media(9) to which had been added 15% human serum to promote growth. Whenever determinations were made, a sample of the media without cells was included. *Chemical determinations* for LD, glutamic oxaloacetic transaminase (GO-T), and glutamic pyruvic transaminase (GP-T) activity were done spectrophotometrically(10,11,12). After clarification by centrifugation, determinations were done on culture media in which the cells had grown, and on cells themselves after they had been broken up by alternate freezing in a dry ice-$CO_2$ bath (–76°C) and thawing in a 37°C water bath 5 times. The frozen and thawed specimens were then centrifuged so that the determinations could be made on the clear supernatant fluid.

*Results. Alteration in LD activity in supernatant fluid of tissue cultures.* Approximately equal numbers of cells from the above mentioned sources were placed in 10 cc of media, inoculated into milk dilution bottles, and placed at 37°C where they settled on the glass surface and began to multiply. One ml of fluid was removed at one- to 2-day intervals, and LD, GO-T, and GP-T activity determinations were made. The same amounts of cells were also set up anaerobically in flat bottomed Erlenmeyer flasks which were then layered with heavy mineral oil to insure preservation of anaerobic conditions. Observation of amount of cell growth was made daily by examining the cells microscopically for the aerobic bottle cultures, and by noting the pH change which occurred in anaerobic cultures. Fig. 1 and 2 show amounts (average of 6 experiments) of LD activity which appeared in the tissue culture fluid following incubation at 37°C up to 26 days. It appears that for the first week of incubation, the fluid bathing the cells showed about the same amount of LD activity, but that in the next 10 days a marked increase of enzymatic activity was noted in the media bathing the malignant cell lines, J-111 and HEP #2, somewhat less in the cell line HEP #1 and the Chang liver, while the first generation amnion and the conjunctiva showed relatively little increase in LD activity, and that of the control media without cells was the lowest. Following the high values, the amount of LD drops quite precipitously at a time when the cells are in a poor nutritional state as evidenced by granularity and sloughing off the glass surface. The behavior of cells and enzymes was the same under anaerobic conditions except that the conjunctival cell line showed a significant increase in LD activity. The GO-T and GP-T determinations done on these same samples failed to vary significantly from day to day, indicating that of the 3 enzyme systems the LD activity was the one reflecting the changes which occurred in the fluid bathing malignant cells.

*Amount of LD in cells and media during growth of cells.* In these experiments a known number of cells was implanted on the side of a test tube, 1.5 cc of culture media was added, and at different points during their growth 2 tubes of each cell line were sacrificed, and the

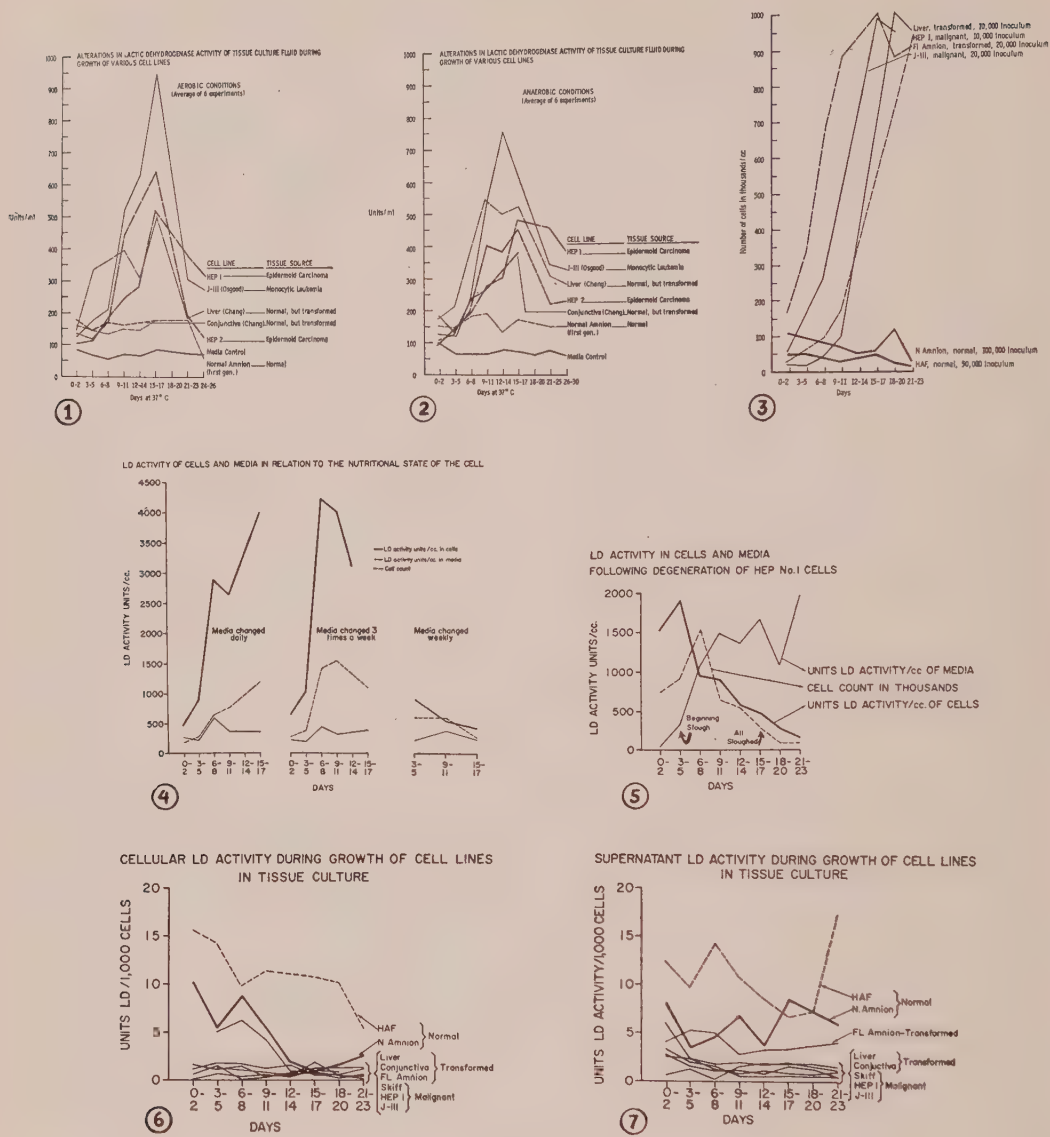


FIG. 1-7.

media in which they had grown was sent for LD activity study. The cells were then detached from the glass surface by trypsin, pooled, sedimented, resuspended in 1 cc of $PO_4$ buffer, and after removal of 0.1 cc for counting, the remaining cells were frozen and thawed and LD activity determined after centrifugation. It was thus possible to compare enzyme activity in the cells with that of the fluid in which they had grown. In one set of experiments the fluid was completely changed in the remaining tubes twice weekly. The following conclusions seem warranted:

1) When cells were implanted in each tube and counts made at frequent intervals (Fig. 3), it can be seen that the amniotic and human fibroblasts failed to multiply to any significant extent, whereas the transformed lines as represented by the FL amnion and the Chang liver, and the known malignant cell lines (J-111 and HEP #1) grew very well even though much smaller inocula had been used. In these experiments the media had been changed twice a week, thus providing

TABLE I. Average Units LD Activity/1,000 Cells in Disrupted Cells and Media during Experimental Periods.

| Cell line | Source | Days of growth 0-8 * | Cells | Media | 9-17 * | Cells | Media | 18-26 * | Cells | Media |
|---|---|---|---|---|---|---|---|---|---|---|
| HAF | Normal | 9 | 13.32 | 12.17 | 8 | 11.34 | 7.89 | 5 | 7.7 | 13.17 |
| Amnion | " | 15 | 7.16 | 4.8 | 17 | 6.4 | 6.14 | 13 | 3.97 | 3.72 |
| FL amnion | Transformed | 8 | .92 | 5.45 | 9 | 1.31 | 3.15 | 7 | 1.73 | 4.08 |
| Liver | " | 12 | 3.47 | 2.49 | 14 | 2.55 | .92 | 10 | 2.29 | .60 |
| Conjunctiva | " | 4 | 3.15 | 2.78 | 7 | 2.58 | 1.67 | 3 | 1.31 | 1.43 |
| HEP #1 | Malignant | 17 | 2.73 | 1.79 | 16 | 1.71 | 1.22 | 12 | 1.32 | 1.03 |
| Skiff | " | 6 | 2.18 | .93 | 6 | 2.52 | 1.79 | 4 | 2.52 | 1.67 |
| J-111 | " | 7 | 5.81 | 1.89 | 9 | 2.76 | .67 | 4 | 1.08 | 1.0 |

* No. of determinations.

good conditions for growth.

2) When LD activity in the frozen and thawed cells and the respective media was compared with the cell count, the results depended to a great extent on whether the media had been changed or not, suggesting that the state of nutrition of the cells played a significant role. Fig. 4 illustrates LD activity observed in the frozen and thawed cells and respective media when 20,000 HEP #1 cells were planted in each tube and the media completely removed and replaced (1.5 cc) from one set daily, from another set 3 times a week, and from still a third set, once a week. The results presented in Fig. 4 show that in the 2 groups with more frequent media change the cells multiplied very well and that sizable activity of LD was found in the cells. Comparatively little LD activity was found in the media, probably because it had been removed and had not had the chance to accumulate. This explanation is suggested by the data obtained from a study of a culture of this same tumor cell in which the media was not changed, and the cells, after a week of vigorous growth, showed large quantities of LD with increasing amounts in the media. As more and more cells sloughed off the glass surface and degenerated, less LD activity was found in the cells themselves, while large activities appeared in the media (Fig. 5). Comparison of the normal, transformed, and malignant cell lines in relation to LD activity in the cells and in the media showed this same effect. When the cells were in a state of good nutrition, significantly more LD activity was present in the cells, no matter from what source they were derived.

3) *Activity of LD/cell in media and cells.* When the amounts of LD per cell are calculated, it appears that the cells from normal sources contain more enzyme activity per cell than do the malignant and transformed cell lines. Table I gives average activity of LD/1,000 cells found in the media and cells during the different periods of growth. This is shown graphically at shorter time intervals in Fig. 6 and 7. In the cells (Fig. 6) human adult fibroblasts, normal amnion, and the J-111 cell lines had more enzyme activity for the first 2 weeks, and thereafter the latter two resemble the other cell lines. In the media (Fig. 7) enzymatic activity per cell is highest throughout for the normal cell lines, while the FL amnion occupies a midway position between them and the others.

In a more direct experiment, growing cells were trypsinized from the glass surface of bottles, counted, and made up to 100,000 in Gey's balanced saline. After being broken up by freezing and thawing, LD activity was determined on the centrifugate. Table II gives the results and confirms the impression that the normal cells contain more of the enzyme activity.

*Discussion.* The observation that LD activity accumulates in fluid bathing malignant cells when grown in tissue culture provides an explanation for the elevated LD activity found in serous effusions. Observations of the condition of the cells have shown that healthy proliferating cells contain large amounts of LD activity, but then they begin to degenerate, less enzyme activity is found

TABLE II. LD Activity Units Released when 100,000 Cells Were Disrupted.

| Cell line | Type of cell | LD activity, units/cc |
|---|---|---|
| HAF | Normal | 1400 |
| Amnion | " | 768 |
| Conjunctiva | Transformed | 480 |
| FL amnion | " | 230 |
| Liver | " | 68 |
| HEP #1 | Malignant | 132 |
| HEP #2 | " | 142 |
| J-111 | " | 140 |
| Skiff | " | 38 |

in the cells themselves and a comparable increase is found in the medium surrounding them. This would seem to be a condition analogous to that found clinically when fluids containing exfoliated cells are found to be the richest in the enzyme activity.

When LD activity for the cell lines derived from normal, transformed, and cancer sources, and grown in tissue culture, are compared, large amounts of activity are found in the known cancer cell lines and in the transformed lines, but little in the normal cell lines. This appears to be a reflection of the rapid growth rate, however, rather than a characteristic of the cell itself, since LD activity per cell is found to be greater in both the normal fibroblasts and amnion cells than in the other cells. How the large amount of LD activity in the fluid bathing the malignant cells accumulates is unknown, but one may speculate that it could diffuse out through the cell wall, or it may escape into the media at the time of cell division.

*Summary.* Normal, malignant, and transformed cell lines have been studied for variations in 3 different enzyme activities, GO-T, GP-T, and LD. Only LD activity changed during the growth period. The enzyme activity both in the cells and in the fluid bathing them was greatly increased in the malignant cell lines and in 2 transformed lines, in contrast to the normal cell lines and the conjunctiva cell line where large quantities did not appear in the media. The increase in LD activity was found to be related to the cellular growth rate rather than to be a characteristic of the cell, since the normal cells in most instances were found to contain more enzyme activity per cell than the malignant ones.

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## Interactions Between Phosphate and Nystatin in *Candida stellatoidea.** (24186)

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Nystatin, an antibiotic produced by *Streptomyces noursei,* inhibits growth of fungi but does not affect growth of bacteria and viruses. It is therefore widely used clinically against a variety of mycotic infections, particularly candidiasis(1). Neither the structure nor mode

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of action of nystatin has been elucidated. It is recognized that this antibiotic inhibits utilization of several substrates normally metabolized by yeasts and molds(2). However nystatin does not inhibit oxidation of citrate, α-ketoglutarate and glutamate. Of particular interest is the observation that nystatin inhibits net uptake of inorganic phosphate(3). This report presents data which suggest that nystatin affects the normal role of phosphate in *Candida stellatoidea*.

*Methods.* Stock cultures of *C. stellatoidea*† were maintained on the complete medium previously described(4). Cells for manometric studies were subcultured continuously as the yeast phase in glucose broth of the following composition: glucose, 60 g; Bacto peptone, 5 g; Bacto yeast extract, 2.5 g; $KH_2PO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 1 g; $(NH_4)_2SO_4$, 2 g; de-ionized water, 1 L. The pH was adjusted to 4.5 prior to sterilization at 121°C for 12 minutes. After incubation at 30°C on a rotary shaker, the 18-hour cultures were harvested by centrifugation and washed twice with cold de-ionized water. The washed cells were then suspended in either 0.067 M phosphate or phthalate buffer which was adjusted to pH 4.5. To each Warburg vessel, 5 mg dry weight of cells were dispensed into the main chamber. Nystatin‡ was added, when indicated, as an aqueous suspension (1500 μg/ml) to the sidearm. Glucose (100 mg/flask) was placed in the second sidearm. All manometric studies were conducted anaerobically by flushing with nitrogen. Respirometers were maintained at 30°C and the shaking rate was 95 strokes/min. The nystatin resistant and the arsenate resistant substrains were obtained from the sensitive parental strain by direct selection(5). The antibiotic was added aseptically to the complete medium as an alcoholic suspension. Mutants resistant to increasing concentrations of nystatin were selected by subculturing survivors on increasing concentrations of the antibiotic. The following increments were employed: 1, 2, 3, 4, 6, 8, 12, and 16 μg/ml.

† Kindly supplied by Dr. Norman F. Conant, Duke University.

‡ Mycostatin, E. R. Squibb & Sons, N. Y.

TABLE I. Inhibitory Effects of Azide and Arsenate on Glucose Fermentation by Nystatin Sensitive and Nystatin Resistant Strains of *Candida stellatoidea*. Warburg vessels contained 5 mg cells (dry wt), 100 mg glucose, phthalate buffer pH 4.5, nitrogen atmosphere.

| Inhibitor | | μl carbon dioxide evolved/hr | |
|---|---|---|---|
| | | Resistant strain | Sensitive strain |
| Sodium azide | $3 \times 10^{-3}$ M | 64 | 70 |
| | $3 \times 10^{-4}$ M | 90 | 91 |
| | $3 \times 10^{-5}$ M | 120 | 124 |
| Monobasic sodium arsenate | $2 \times 10^{-2}$ M | 90 | 76 |
| | $10^{-2}$ M | 105 | 84 |
| | $5 \times 10^{-3}$ M | 138 | 104 |
| | $10^{-3}$ M | 150 | 123 |
| | $10^{-4}$ M | 150 | 145 |
| Control | | 150 | 150 |

Similarly growth from the complete medium containing 0.01 M $NaH_2AsO_4$ was plated on complete medium containing 0.02 M arsenate. Resistant strains were subcultured at least once in the absence of inhibitor prior to each experiment.

*Results.* The effect of metabolic poisons on glucose fermentation by parental and nystatin resistant strains was determined. As shown in Table I, sodium azide, at concentration of 0.003 M, reduced fermentative production of $CO_2$ by 50% whereas 0.00003 M azide reduced $CO_2$ evolution by 20%. In this instance the nystatin sensitive and resistant strains responded identically.

Arsenate likewise inhibited glucose fermentation by both the nystatin sensitive and nystatin resistant strains. Unlike azide, arsenate inhibited the parental strain more than the nystatin resistant strain. At a concentration of 0.001 M arsenate, $CO_2$ production by the nystatin resistant strain was not affected, whereas under the same conditions $CO_2$ production by the parental strain was reduced 15%.

The inhibitory effect of 0.01 M arsenate on glucose fermentation by the nystatin sensitive parental strain was completely reversed by 0.067 M phosphate buffer (Table II). This is consistent with the concept that arsenate inhibits oxidative phosphorylation of 3-phosphoglyceraldehyde in the Embden-Meyerhof scheme. Additional data, not presented here, plotted according to Lineweaver

TABLE II. Reversal by Phosphate of Inhibitory Effects of Arsenate and Nystatin on Glucose Fermentation by the Parental Strain. Warburg vessels contained 5 mg cells (dry wt), 100 mg glucose, either phthalate buffer or phosphate buffer pH 4.5, nitrogen atmosphere.

| Inhibitor | $\mu$l $CO_2$/hr | |
|---|---|---|
| Monobasic sodium arsenate | no phosphate | .067 M phosphate |
| $2 \times 10^{-2}$ M | 88 | 134 |
| $10^{-2}$ M | 105 | 169 |
| $5 \times 10^{-3}$ M | 145 | 202 |
| Control | 150 | 150 |
| Nystatin | no phosphate | .5 M phosphate |
| 60 $\mu$g/ml | 96 | 107 |
| 40 " | 110 | 136 |
| 20 " | 117 | 150 |
| Control | 150 | 150 |

and Burk, confirmed the competitive nature of the arsenate-phosphate interaction.

These findings suggested that inhibition of glucose fermentation caused by nystatin might be reversed by the presence of phosphate. Although 0.067 M phosphate was not effective in this respect, addition of 0.5 M phosphate completely canceled the 20% inhibition produced by 20 $\mu$g nystatin/ml.

As shown previously, the nystatin resistant strain was less susceptible to arsenate than its nystatin sensitive counterpart. Moreover inhibition of glucose fermentation produced by arsenate and nystatin could be reversed by appropriate concentrations of phosphate. On the basis of these findings it was postulated that an arsenate resistant mutant might have increased resistance to nystatin. Indeed experiments confirmed that glucose fermentation by the arsenate sensitive parental strain was reduced 20% by 20 $\mu$g nystatin/ml whereas the arsenate resistant strain was not affected by this concentration of nystatin (Table III).

The significance of the manometric results, as correlated with the fungistatic property of the antibiotic, was determined by growth experiments. Phosphate, added to complete medium to a maximal concentration of 0.5 M, did not reduce the fungistatic activity of nystatin (4 $\mu$g/ml). However growth experiments confirmed that the nystatin resistant strain was less susceptible to arsenate than the parental strain. Moreover the arsenate resistant strain was more resistant to nystatin than its arsenate sensitive counterpart.

*Discussion.* One of the pertinent findings of this study was that a greater concentration of nystatin was required to inhibit growth of *Candida stellatoidea* in glucose broth than in the complete broth. This is consistent with the report that glucose and peptone reverse nystatin inhibition(6). It is of interest that growth of the *C. stellatoidea* strain we used was inhibited by as little as 1.3 $\mu$g nystatin/ml in the complete medium. After several subcultures in the presence of increasing concentrations of nystatin, a strain resistant to 16 $\mu$g/ml was obtained. Because it is reportedly difficult to isolate mutants resistant to nystatin, it is noteworthy that the resistant strain described here developed such a high degree of resistance(7).

Our findings show that resistance to nystatin was accompanied by resistance to arsenate. Similarly selected resistance to arsenate was associated with increased resistance to nystatin. Such results suggest that nystatin may affect the oxidative phosphorylation of 3-phosphoglyceraldehyde to 1,3-diphosphoglyceric acid. This is consistent with the recognized mode of action of this inhibitor. Nystatin could therefore exert its inhibitory effect 1) by complexing with 3-phosphoglyceraldehyde, 2) by binding inorganic phosphate essential for oxidation, 3) by interfering with diphosphopyridine nucleotide, or 4) by inactivating the enzyme itself. In as much as inhibition of glucose fermentation by nystatin is reversed by phosphate, it is conceivable that nystatin may act by binding inorganic phosphate. It is pertinent in this

TABLE III. Inhibitory Effect of Nystatin on Glucose Fermentation by Parental and Arsenate Resistant Strains of *Candida stellatoidea*. Warburg vessels contained 5 mg cells (dry wt), 100 mg glucose, phosphate buffer pH 4.5, nitrogen atmosphere.

| Nystatin | $\mu$l $CO_2$/hr | |
|---|---|---|
| | Sensitive strain | Resistant strain |
| 100 $\mu$g/ml | 18 | 80 |
| 60 " | 79 | 113 |
| 20 " | 120 | 158 |
| Control | 150 | 150 |

regard to note that nystatin activity is reduced by cysteine and that cysteine reactivates triosephosphate dehydrogenase which has been inactivated by oxidation. These latter observations may have a causal relationship.

The manometric studies reported here have been concerned only with fermentation, in spite of the fact that it has been previously shown that respiratory reactions are also affected. Therefore the effects of nystatin reported herein are but one of several possible manifestations. It is premature on the basis of these data to assert that the primary mode of action of nystatin concerns oxidative phosphorylation. Certainly this is an important site and the correlation between manometric findings and growth studies constitutes circumstantial evidence that this may be the case.

*Summary.* Glucose fermentation by *Candida stellatoidea* was inhibited by sodium arsenate and by the fungistatic antibiotic, nystatin. Mutants selected for resistance to one inhibitor simultaneously developed increased resistance to the other inhibitor. The inhibitory effects of arsenate and nystatin on glucose fermentation by the sensitive parental strain were reversed by appropriate concentrations of phosphate.

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## Studies on Erythropoiesis IX. Mechanism of Decreased Erythropoiesis In Experimental Polycythemia. (24187)

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That increased erythropoiesis following production of acute anemia is mediated on a hormonal basis was first suggested by Carnot and Deflandre(1). In recent years, numerous investigators have demonstrated the appearance of a humoral erythropoietic-stimulating factor in anemic animals(2-9). Fried, Plzak, Jacobson, and Goldwasser(10) have presented evidence supporting the hypothesis that the rate of erythropoiesis is determined by the amount of erythropoietin in plasma at all times. It has long been known that experimentally-induced polycythemia depresses erythropoiesis(11-15). That decreased concentrations of erythropoietin are responsible for depression of erythropoiesis in transfusion-induced polycythemia was first suggested by Fried *et al.*(10) and Jacobson *et al.*(16). Demonstration of decreased concentrations of erythropoietin in plasma of transfusion-induced plethoric animals and human beings is as important in establishing validity of the concept of a humoral control of erythropoiesis as is demonstration of increased erythropoietin concentrations in plasma obtained from anemic donors. In this paper, the decline in reticulocyte count and incorporation of $Fe^{59}$ into newly-formed red cells of polycythemic rats, and degree of decline in relation to severity of polycythemia are reported. In addition, response of these polycythemic animals to anemic plasma and cobaltous chloride is described.

*Methods and materials.* Rats of the Sprague-Dawley strain were used in this study. Animals in last experiment were hypophysectomized by Hormone Assay Laboratories of Chicago. Blood was removed from donor animals by cardiac puncture, using heparin as anticoagulant. Intravenous trans-

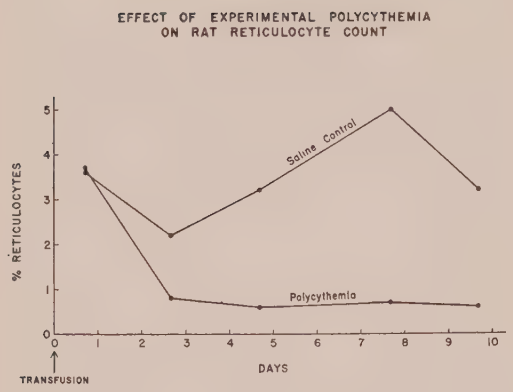


FIG. 1. Effect of experimentally-induced polycythemia on No. of reticulocytes in the rat.

fusions consisted of whole blood or 50% red cells resuspended in saline. Intraperitoneal injections consisted of 80% red cells suspended in saline. In the last 3 experiments, periodic hemoglobin and reticulocyte counts were determined on blood obtained from tail vein. In all other studies, blood was obtained by cardiac aspiration at time of sacrifice. Hemoglobin values were determined by measurement of oxyhemoglobin as described by Evelyn(17). Reticulocytes were counted by direct smear method, using brilliant cresyl blue stain without counter-stain. Rate of erythropoiesis was then determined as follows(18): $Fe_3^{59}$ citrate (1 to 2 $\mu$c) was injected into tail vein of animals, and 16 hours thereafter 1 ml of blood was obtained by cardiac puncture. Standards were prepared at time the isotope was administered to animals. Activity of standards and the blood was determined by counting in a well-type scintillation counter. The amount of radioactivity in the entire circulating red cell mass was calculated by multiplying the activity per ml of blood by the blood volume and was expressed as per cent of injected dose of $Fe^{59}$ in peripheral red cells. Blood volumes of rats with varying degrees of polycythemia were determined in preliminary experiments by the method of Berlin *et al.*(19). Plasma referred to as "anemic" rat plasma was obtained from rats whose hematocrit had been reduced to 25% by 3 daily cardiac aspirations. Human plasma was obtained from normal donor and a donor with idiopathic aplastic anemia, using heparin as anticoagulant. Extracts of human plasma were prepared by the method of Borsook *et al.*(20).

*Results. I. Decrease of reticulocyte count and red cell incorporation of $Fe^{59}$ following intraperitoneal injection of blood.* Rats weighing 250-280 g were made polycythemic by single intraperitoneal injection of packed cells equal to 4% body weight, and control animals were given the same volume of normal saline. Reticulocyte counts and red cell $Fe^{59}$ incorporation were determined in separate groups of 5 animals at the times indicated in Fig. 1 and 2. No change in reticulocyte count was noted 17 hours after administration of blood, but at 2 days and thereafter a marked reduction in number of reticulocytes was noted (Fig. 1). Similarly, incorporation of $Fe^{59}$ was reduced at 4 days and fell steadily, reaching its low value of 5% when the tracer was given 9 days after the animals were made polycythemic (Fig. 2).

*II. Effect of varying degrees of polycythemia on number of reticulocytes and incorporation of $Fe^{59}$.* Rats weighing 150 to 200 g were given intraperitoneal injections as indicated in Table I. $Fe^{59}$ was administered 8 days later, and the following morning incorporation of iron and number of reticulocytes was obtained. Only a modest rise in hemoglobin was obtained following administration

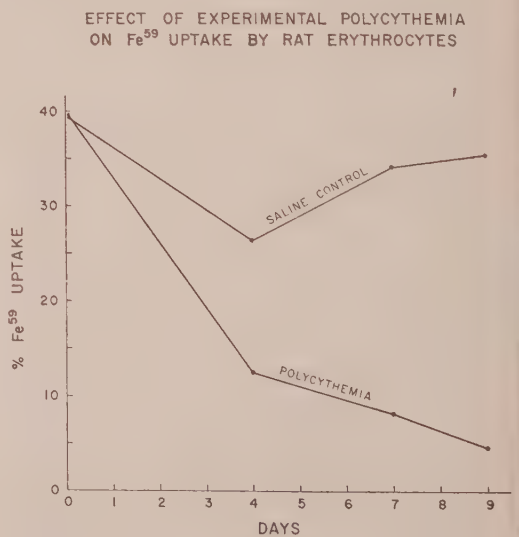


FIG. 2. Effect of experimentally-induced polycythemia on incorporation of $Fe^{59}$ into newly-formed red blood cells in the rat.

TABLE I. Depression of Erythropoiesis following Experimental Polycythemia.

| Vol inj.* | Packed cells 2% | Saline 2% | Packed cells 4% | Saline 4% | Packed cells 8% | Saline 8% |
|---|---|---|---|---|---|---|
| No. of animals | 5 | 5 | 5 | 4 | 5 | 4 |
| Hemoglobin | 15.6 | 14.6 | 16.7 | 13.6 | 21.2 | 14.8 |
| Reticulocyte (%) | 1.1 | 5.6 | .2 | 6.3 | .1 | 5.1 |
| $Fe^{59}$ uptake (%) | 19 | 48 | 8 | 48 | 6 | 48 |

* Vol inj. in ml is numerically equal to 2, 4, or 8% of animals' body wt in g.

of smallest amount of blood, but a definite reduction in number of reticulocytes and incorporation of $Fe^{59}$ was obtained (Table I). It appears that packed cells in 4% of animal's body weight, elevating the hemoglobin to only 16.7 g %, are almost as efficacious in suppressing reticulocyte production and red cell $Fe^{59}$ incorporation as twice this volume of packed cells, an amount producing a far more dramatic elevation of hemoglobin.

*III. Effect of rat plasma and heat-denatured extract of human plasma on incorporation of $Fe^{59}$ into erythrocytes of the polycythemic rat.* Rats weighing 75 to 125 g were given packed red cells, in milliliters equal to 4% of body weight in g. On 11th and 12th days after infusion, these animals received 2 ml of plasma or plasma extract given subcutaneously (Fig. 3). Radioiron uptake by the erythrocytes was then determined, the tracer dose being administered on the 13th day after establishment of polycythemia. Approximately 6-fold increases in $Fe^{59}$ incorporation resulted from administration of anemic rat plasma or the heat-denatured extract of plasma from an anemic human patient.

*IV. Effect of cobalt on number of reticulocytes and uptake of $Fe^{59}$ in polycythemic rat.* Rats weighing 140 to 170 g were made polycythemic by intraperitoneal injection of packed cells in the volume in ml equal to 4% of their body weight in g. On the third day after injection, the animals were divided into 2 groups by random selection. One group received cobaltous chloride subcutaneously and the other group received normal saline. On the 12th day, $Fe^{59}$ was administered and its incorporation was determined 16 hours later. Reticulocyte and hemoglobin values are recorded in Table II. Although the animals were fortuitously divided in such a manner that the saline control group on all occasions had slightly lower average hemoglobin values than the animals receiving cobalt, reticulocyte averages were higher in the animals receiving cobalt. This difference became more striking with the passage of time. Further, incorporation of $Fe^{59}$ into newly-formed red cells in polycythemic animals was increased from an average value of 5% in the rats receiving saline to an average value of 32% in the rats receiving cobalt.

*V. Effect of multiple transfusions on reticulocyte count in the polycythemic rat.* Four rats weighing 150 to 185 g were made and maintained polycythemic for 48 days by multiple transfusions in an effort to obtain com-

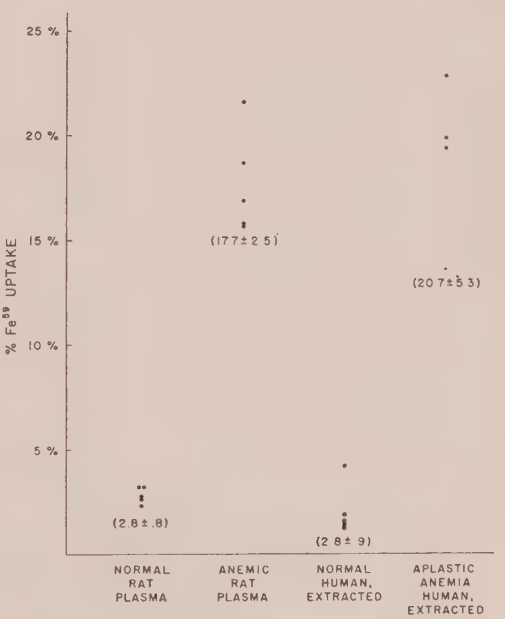


FIG. 3. Effect of normal and anemic rat plasma and normal and anemic human plasma (heat-denatured extract) on incorporation of $Fe^{59}$ into newly-formed red blood cells in polycythemic rats. Numbers enclosed in parentheses are avg values ± one stand. dev.

TABLE II. Reticulocyte Response following $Co^{++}$ in the Polycythemic Rat.

| Day | Control (5 rats) Hb | Control (5 rats) Retic. | Control (5 rats) Injection | Cobalt (4 rats) Hb | Cobalt (4 rats) Retic. | Cobalt (4 rats) Injection |
|---|---|---|---|---|---|---|
| 3 | | | Saline, 1 cc/day | | | Cobalt, 5 $\mu$M/day |
| 6 | 17.8 | .3 | | 18.9 | .7 | |
| 8 | 17.9 | .3 | | 18.5 | 1.6 | |
| 10 | 17.1 | .1 | Saline, 1 cc B.I.D. | 18.0 | 1.7 | Cobalt, 5 $\mu$M B.I.D. |
| 12 | 17.2 | .3 | | 18.2 | 2.5 | |

plete suppression of erythropoiesis as indicated by a complete disappearance of reticulocytes from the peripheral blood. At least 4 days elapsed after each transfusion before counts were repeated in order to permit maturation of transfused reticulocytes. A 50% suspension of red cells in saline was used after the first week in an effort to eliminate stimulation of erythropoiesis by erythropoietin present in normal plasma. Every reticulocyte value given in Table III was determined by examining 10,000 cells after the polycythemia was established. It was not possible to rid the rats completely of reticulocytes, although while the hemoglobin was maintained above 20 g, it frequently was necessary to count 10,000 red cells on a smear to find a single reticulocyte (Table III).

*VI. Effect of polycythemia on reticulocyte count of hypophysectomized rat.* Six days after hypophysectomy, the reticulocyte count of 9-week-old 120-140 g rats averaged .06% and average hemoglobin was 18.9 g %. None of 7 rats was completely free of reticulocytes, however. Two daily intravenous injections of blood, totaling 14 ml per rat, were given and 8 days later the average hemoglobin was 23.4 g %. The average reticulocyte count was .006%, and in 4 of the 7 rats, the count was 0.00%, no reticulocytes being seen in 10,000 cells.

*Discussion.* The present investigation has been directed toward the accumulation of evidence that decreased erythrocyte formation in transfusion-induced polycythemia is a result of lowered erythropoietin titers. In this regard, it is of interest to note that the high titer of erythropoietin associated with some anemias in human beings declines when, as a consequence of transfusions, the hemoglobin increases(21,22).

Slight increases in circulating levels of hemoglobin reduce but do not completely inhibit erythrocyte production since reticulocyte production and iron uptake were reduced but not completely inhibited 8 days after transfusion. This reduction was greater when associated with increased plethora, but it is of interest to note that the reduction in erythropoiesis, as measured by the number of reticulocytes, appeared to be of about the same magnitude when the hemoglobin was 16.7 g % as it was when the value was 21.2 g %. Since none of the animals in the latter group was free of reticulocytes, we then attempted to eliminate the reticulocyte count in the rat with multiple transfusions, as has been accomplished in mice(16). This reticulocyte count was easily depressed to 0.00% in several hypophysectomized rats made polycythemic. Although marked depression of the reticulocyte count was accomplished in the normal rat made polycythemic by transfusion, in no instance was a complete absence of reticulocytes found. It appears likely to us that the poly-

TABLE III. Reticulocyte Suppression in Prolonged Transfusion-Induced Polycythemia.

| Day | 1 Hb | 1 Retic., % | 2 Hb | 2 Retic., % | 3 Hb | 3 Retic., % | 4 Hb | 4 Retic., % |
|---|---|---|---|---|---|---|---|---|
| 0 | 11.6 | 5.7 | 9.6 | 6.4 | 10.4 | 5.2 | 12.3 | 6.9 |
| 15 | 21.6 | | 21.6 | .03 | 20.9 | .12 | 21.4 | .08 |
| 25 | 22.7 | .03 | 24.0 | .02 | 22.8 | .03 | 23.4 | .10 |
| 29 | 21.4 | .02 | 24.8 | .01 | 22.7 | .02 | 20.8 | .01 |
| 48 | 21.2 | .02 | 19.4 | .04 | 19.4 | .04 | 21.4 | .03 |

Transfusions given on days 0, 4, 7, 11, 18, 19, 20, 29, 40, 41.

cythemic rats do not deliver enough oxygen to the site of production of erythropoietin to completely suppress its formation, but after hypophysectomy the metabolic rates and oxygen requirements are depressed, hence in the polycythemic hypophysectomized rat the additional oxygen made available after transfusion is sufficient to accomplish this purpose.

The increased uptake of $Fe^{59}$ in the polycythemic rats following 2 injections of anemic, but not normal, rat plasma suggests that reduction of erythropoiesis in the plethoric rat is mediated through reduction of erythropoietin titers. This concept is fortified by the finding that the extract of plasma obtained from an anemic human patient following removal of the bulk of the protein by heat-denaturation also increases the incorporation of iron in plethoric rats, while a similarly prepared extract of normal human plasma fails to do so. Further evidence for this concept is found in the cobalt-induced reversal of the depression of erythropoiesis in the polycythemic rat, since it has been demonstrated by Goldwasser and his co-workers(23) that cobalt-induced polycythemia appears to be a consequence of increased erythropoietin production.

*Summary.* 1) Reduction of erythropoiesis in rats rendered polycythemic by intraperitoneal infusion of packed red cells bears a relation to the degree of plethora produced. Plasma from anemic rats, a heat-denatured plasma extract from a patient with aplastic anemia, and subcutaneous cobaltous chloride all stimulate erythropoiesis in the polycythemic rat. These observations are consistent with the theory that decreased erythropoiesis in the polycythemic rat is the immediate consequence of a decreased titer of erythropoietin in these animals, and constitute further support for the theory of the humoral regulation of erythropoiesis. 2) In these experiments it was not possible to suppress erythropoiesis completely, as measured by the reticulocyte count, in normal rat by transfusion, although complete suppression was readily accomplished in transfused hypophysectomized rat.

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# Antigens of Rabbit Semen.* (24188)

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It has long been known that spermatozoa are antigenic and possess a high degree of immunological specificity(1-5). Strong antigenicity was also reported for seminal plasma of man(4-6) and several animal species(3,5, 7). Both for spermatozoa and for seminal plasma evidence was obtained that more than one antigenic entity is involved. That spermatozoa and seminal plasma have in common powerful and seemingly preponderant antigens has recently been established both for man(4) and guinea pig(5). In man, at least, indications are that the common antigens do not have their origin in the testes, but in the adnexal glands of the male genital tract. The antigenic material, however, is so firmly adherent to spermatozoa, that it has hitherto been impossible to separate it from these cells by physical means. Further study of the immunological relationship between spermatozoa and seminal plasma seemed to be indicated, with emphasis on the search for the site of production of these antigens.

*Methods.* Dutch belted rabbits purchased from Rockland Farms served as semen donors. Semen was obtained by means of the artificial vagina, first developed by Macirone and Walton(8) and introduced in this country by Gregoire, Bratton and Foote(9)†. Rubber balloons from Anderson Co., Akron, cut at the tip, provided satisfactory linings. Procedures of complement fixation test, sperm-agglutination, and hemagglutination with tanned erythrocytes were identical with those described before(4). The Ouchterlony technic(5,10) was used for demonstration of antigen-antibody reaction by agar-diffusion. Controls with antigen alone and antigen plus pre-immunization serum were included in the tests and always found non-reactive. Rabbit semen contains spermatozoa in the order of 200 million/ml. Small numbers of large cells with round nuclei and numerous spherical bodies of approximate size of cocci are also present. Their nature is unknown. It is worth noting that these spherical bodies are not affected in any visible way by addition of immune serum in the slide agglutination test with spermatozoa. Immune sera of high antibody titer were obtained in guinea pigs after 2 intramuscular injections at 2 week interval of 0.5 ml of a mixture of equal parts of Freund's adjuvant (Difco) and either seminal plasma (diluted 2/5 with physiological saline solution) or washed spermatozoa in saline (adjusted to contain 10 million cells/dose.) Serum was collected 3-4 weeks after last injection. Sera of 4-6 guinea pigs were pooled after preliminary individual tests for titer. Suspensions of spermatozoa in physiological saline solution were treated in the Magnetostriction Oscillator (Raytheon, 9 kilocycles, 50 W). By alternating centrifugation at low and high speeds 3 fractions were obtained, namely (a) spermatozoal heads and a few detached tails; (b) tails plus the spherical particles mentioned before; and (c) a clear supernatant fluid.

*Results.* Complement fixation in serum dilutions varying between 1/100 and 1/1600 was obtained. Anti-seminal plasma and anti-spermatozoal sera were indistinguishable in reactivity with the 2 antigens. Complement fixation was observed with seminal plasma in dilutions varying between 1/1600 and 1/12800 or slightly better (Table I).

Our immune sera against rabbit seminal plasma and spermatozoa did not show cross reactivity with human seminal products and vice versa. These observations further confirm data on species specificity of seminal antigens in the literature, including the recent data of Katsh(11). The occasional findings of this author suggesting slight cross-reactivity in maximally sensitized guinea pigs merit further critical exploration.

* Supported by Grant from N.I.H., U.S.P.H.S.

† We are most grateful for the opportunity of learning their procedure at Cornell Univ. Agric. Exp. Station.

TABLE I. Fixation of Complement by Guinea Pig Immune Serum (Diluted 1/20) and Antigens (Rabbit).

| Antigen dilution | A Seminal plasma 1/200 | | B Spermatozoa $10^6$/0.1 ml | | C Testis 1/10 | | D Epididymis 1/10 | | E Seminal vesicle 1/10 | | F Seminal vesicle content 1/10 | | G Prostate 1/10 | | H Kidney 1/10 | | I Liver 1/10 | | J Serum 1/100 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Anti-sem. | Anti-sp. | Anti-sem. | Anti-sp. | Anti-sem. | Anti-sp. | Anti-sem. | Anti-sp. | Anti-sem. | Anti-sp. | Anti-sem. | Anti-sp. | Anti-sem. | Anti-sp. | Anti-sem. | Anti-sp. | Anti-sem. | Anti-sp. | Anti-sem. | Anti-sp. |
| 1/1 | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | — | — | — | — |
| 1/2 | " | " | " | " | " | " | " | " | " | " | " | " | " | 3+ | " | " | — | — | — | — |
| 1/4 | " | " | " | " | " | " | " | " | " | " | " | " | 3+ | — | 2+ | 2+ | — | — | — | — |
| 1/8 | " | " | " | " | 3+ | " | 1+ | 3+ | " | " | " | " | — | — | — | — | — | — | — | — |
| 1/16 | " | " | " | " | — | 3+ | — | — | " | — | " | " | — | — | — | — | — | — | — | — |
| 1/32 | " | " | " | " | — | — | — | — | 3+ | — | " | " | — | — | — | — | — | — | — | — |
| 1/64 | " | 2+ | 3+ | 4+ | — | — | — | — | — | — | " | " | — | — | — | — | — | — | — | — |
| 1/128 | 1+ | (+) | 1+ | 1+ | — | — | — | — | — | — | " | 3+ | — | — | — | — | — | — | — | — |
| 1/256 | — | — | — | — | — | — | — | — | — | — | 1+ | — | — | — | — | — | — | — | — | — |

Antiserum and antigen controls, also antigen plus normal rabbit serum negative.
Anti-sem. = Anti-seminal plasma serum; Anti-sp. = Anti-spermatozoal serum.
4+, no hemolysis; 3+, slight (approx. 30%) hemolysis; 2+, moderate (approx. 50%) hemolysis; 1+, strong (approx. 70%) hemolysis; (+), almost complete; —, complete hemolysis.

If heavy suspensions of thrice washed rabbit spermatozoa (100 million/ml or more) in saline are kept in the refrigerator 1 to 8 weeks, the clear supernate obtained by centrifugation is reactive in complement fixation test with anti-seminal plasma or anti-spermatozoal sera in dilutions of 1/64 to 1/128. The spermatozoa of the sediment showed no measurable loss of reactivity.

The fractions obtained after sonic vibration of spermatozoa gave complement fixation to approximately equal extent with anti-seminal plasma and antispermatozoal sera. No immunological differentiation was observed.

Attempts to use methods of adsorption failed, due to development of anticomplementary properties. The anticomplementary action could not be diminished by 2 hours' centrifugation at 33,000 g (16,000 rpm in Lourdes high speed angle centrifuge).

High organ specificity of immune sera is exemplified by data given in Table I. The organ extract employed in these tests was prepared as previously described for material of human origin(4). In striking contrast to the slight cross-reactivity of organ extracts and lack of cross reactivity with rabbit serum, the fluid obtained from seminal vesicles was reactive in dilutions comparable with those of seminal plasma. This fluid was obtained by removal of the content from seminal vesicles (which are rather large in the rabbit) with a fine hypodermic needle and syringe. Some turbidity was removed by centrifugation. Under the microscope the sediment showed numerous spherical bodies similar to those described above for semen, and a very few spermatozoa. The clear supernate was not anticomplementary.

Indications of a difference in reactivity between anti-seminal plasma and the anti-spermatozoal immune sera were also absent in hemagglutination tests with tanned human erythrocytes on which seminal plasma was adsorbed (for technical data see 4 and 12). Agglutination was observed in serum dilutions ranging up to 1/40,000. No systematic difference was seen between the 2 kinds of immune sera.

Tanned erythrocytes treated with the con-

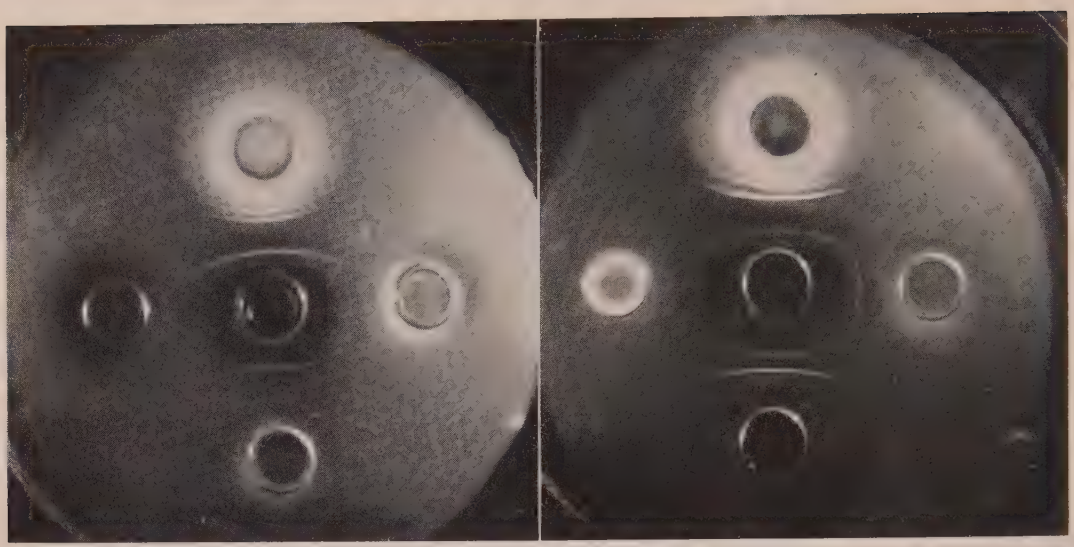

FIG. 1. Centers: Anti-rabbit seminal plasma serum (undiluted). Outer troughs: Antigens undiluted, rabbit. *Left frame:* top, seminal plasma; right, testis; bottom, epididymis; left, blood serum. *Right frame:* top, seminal plasma; right, seminal vesicle, wall; bottom, seminal vesicle, content; left, prostate.

tent of seminal vesicles were agglutinated by our immune sera in dilutions up to 1/1600. However, tanned erythrocytes treated with various organ extracts were not agglutinated by sera in dilutions of 1/25.

Both anti-seminal plasma and antispermatozoal immune sera strongly agglutinated spermatozoa from whole fresh semen and also spermatozoa thrice washed with physiological saline solution. The slide agglutination method(4,13) was used. With fresh semen, serum dilutions up to 1/4096 gave definite agglutination. With washed spermatozoa, the titers were lower, ranging from 1/16 to 1 512. These findings differ from those of Smith (3) who observed higher agglutination titers with washed spermatozoa than with fresh semen. Again, no difference could be detected in the behavior of the 2 kinds of immune serum employed. Agglutination was essentially of the "tail" type, as also reported by Smith(3).

In the absence of spermatozoal antigen in soluble form, immune precipitation by the agar diffusion method is at a disadvantage for purposes of testing spermatozoal antigen. However, the method yielded 2 contributions of interest for our problem: Antiseminal plasma and anti-spermatozoal sera gave the same precipitation lines with seminal plasma, namely 2 heavy ones close to and curved toward the trough containing the antigen and 3 (2 of them very close together) near and curved toward the antiserum (Fig. 1). Thus by this technic also, the 2 kinds of antiserum remain indistinguishable in their reactivity with seminal plasma. Secondly, antiseminal plasma immune serum can be completely exhausted by spermatozoa, and anti-spermatozoal serum, in turn, by seminal plasma as far as reactivity in the Ouchterlony test (with seminal plasma as antigen) is concerned. The value of this evidence is somewhat restricted by the fact that the agar diffusion method is less sensitive than the complement fixation test.

The data obtained by complement fixation method on organ specificity of immune sera were supplemented in an illuminating way by the results with the agar diffusion technic (Fig. 1). As far as cross reactions with organ extracts can be demonstrated, they are related to one single precipitation line, namely the second one (counted from trough containing antiserum). There is one important exception; with the extract from seminal vesicle, all 3 lines close to the antiserum containing trough are produced. The content of

the seminal vesicles produced 4 lines obtained with seminal plasma, *i.e.*, all of the reactions of seminal plasma except the one indicated by innermost (nearest to antiserum trough) weak line. Serum, kidney and liver extracts did not produce lines of precipitation.

*Conclusions and summary.* 1) Comparison of data with those on human semen presented previously(4) shows a close similarity in the immunological properties of seminal products of man and rabbit. In both species, seminal plasma and spermatozoa contain powerful antigens, so closely related that they have resisted differentiation. 2) These antigens are highly species and organ specific. Such cross-reactions as could be obtained, according to evidence provided by agar diffusion method, are due to one of the 5 components demonstrable by this procedure. The multiplicity of antigenic components corresponds with that previously shown in man and guinea pig (4-6). 3) Data obtained from human semen specimens lacking spermatozoa suggested that the antigenic material does not originate with spermatozoa. This view is further strengthened by the low degree of cross reactivity of rabbit sera with extracts from testes and epididymis, and, still more strongly, by evidence that extracts of seminal vesicle wall contain some antigenic components reactive with anti-seminal plasma and anti-spermatozoal immune sera. The seminal vesicle fluid of the rabbit contains in abundance nearly all antigenic components demonstrable with our sera. 4) The effective antigens found in seminal plasma and spermatozoa of semen appear to originate in the seminal vesicle.

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## Inhibition of Carbonic Anhydrase: Effect on Tissue Gas Tensions in the Rat* (24189)

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The established importance of carbonic anhydrase in $CO_2$ transport implies impairment in $CO_2$ elimination when the enzyme is inhibited. This controversial problem, has been discussed, and evidence presented for impaired $CO_2$ transport with resultant retention of this gas following carbonic anhydrase inhibition in the dog(1). The evidence presented, however, did not demonstrate a rise in venous or tissue $CO_2$ tension following inhibition of carbonic anhydrase, a change implicit in the mechanism of $CO_2$ retention from any cause. Such demonstration was not possible because usual technics for measurement of venous $CO_2$ tension are inapplicable in the presence of carbonic anhydrase inhibition. When carbonic anhydrase is inhibited a dis-

* This work was supported by Research Grant from Council on Neurological Diseases and Blindness.

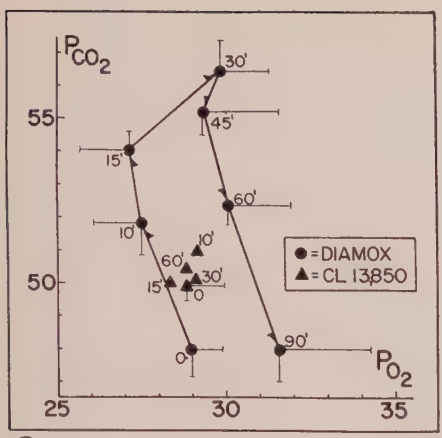
equilibrium develops between $CO_2$ and carbonic acid and hence between $CO_2$ and bicarbonate. Measurements of $CO_2$ tension in blood performed *in vitro* allow time for equilibration to occur in the absence of enzyme activity and hence give false values for $P_{CO_2}$ when carbonic anhydrase is inhibited. In addition, use of the Henderson-Hasselbalch equation is inapplicable under circumstances of carbonic anhydrase inhibition because its derivation is predicated on the near instantaneous equilibrium between $CO_2$ and carbonic acid made possible by carbonic anhydrase(2). In contrast to difficulties in measurement of $P_{CO_2}$ in venous blood, arterial $CO_2$ tension can be estimated by analysis of alveolar air, since lungs afford a natural tonometer whose $CO_2$ tension reflects that of the gas as it existed in arterial blood leaving the lungs. The composition of an enclosed, gas-filled space within the body is known to approach equilibrium with venous blood perfusing it, hence its gas tensions approach those of adjacent tissues(3,4). Analysis of a well established, vascularized subcutaneous gas pocket in the rat was a convenient means of estimating gas pressures of venous blood perfusing the pocket(4). We report here the use of an artificially constructed tonometer on the venous side of the circulation which allows estimation of $CO_2$ tension of tissue and venous blood under peculiar circumstances of carbonic anhydrase inhibition when such measurements are otherwise impossible.

*Methods.* A. *Effect of carbonic anhydrase inhibition on gas composition of subcutaneous pockets.* Subcutaneous gas pockets were established in 10 adult Wistar rats by injection of 20 cc of air into the subcutaneous tissue of the interscapular fossa(4). After 2 weeks, when control analyses indicated a steady state, one-half the rats were injected intraperitoneally with 50 mg/kg body weight of acetazolamide† in a volume of 2 cc at a pH of 10. The other rats were given a control substance, CL 13,850‡ (2-acetylamino-1,3, 4-thiadiazole-5-sulfon-*t*-butylamide), by the same route in equal molar dosage at same pH. This substance is very similar to acetazolamide in its physical properties such as molecular weight and acid dissociation constants but has only $4 \times 10^{-5}$ times the inhibitory effect on carbonic anhydrase. Gas samples were obtained on all rats at intervals to 90 minutes after injection and analyzed using a Scholander micro gas analyzer. Measurement of blood carbonic anhydrase activity before and 30 minutes after injection of the 2 substances was carried out in comparable group of 6 rats (5). B. *Effect of carbonic anhydrase inhibition on pulmonary ventilation.* Six Wistar rats weighing 250-300 g were anesthetized with pentobarbital injected intraperitoneally in dose of 29 mg/kg. The tracheae were cannulated and ventilation measured by a recording microspirometer containing oxygen. $CO_2$ was absorbed by a sodium hydroxide wick within the spirometer. After ventilation had become steady acetazolamide was injected intraperitoneally in 50 mg/kg in volume of 1 cc. Ventilation was measured thereafter at intervals to 30 minutes. Measurement was made for one minute, the spirometer then flushed with oxygen. The effect of anesthesia alone on ventilation was measured by this same method using intraperitoneal injection of 1 cc .85 N saline as control. *C. Effect of carbonic anhydrase on $CO_2$ content of arterial blood.* $CO_2$ content of arterial blood as well as gas tensions of subcutaneous pockets were measured in 16 other rats, following administration of acetazolamide and CL 13,850. After control samples of well established pockets indicated a steady state, the drugs were injected intraperitoneally each to ½ the animals in dose described. Thirty minutes later, (the time of maximal change), the pockets were reanalyzed, then animals were struck on head. The chest was quickly opened and 2-3 ml of blood were withdrawn anaerobically from left ventricle which had not yet stopped beating. The time from the blow to completion of sampling was standardized to 1½ minutes. The hematocrit was measured by a micro-pipette technic.

*Results.* A. *Gas tensions in pockets.* The $CO_2$ tension of gas pockets rose progressively

† Diamox was supplied through the courtesy of Lederle Lab. Division, American Cyanamid Co.

‡ Supplied through same company.



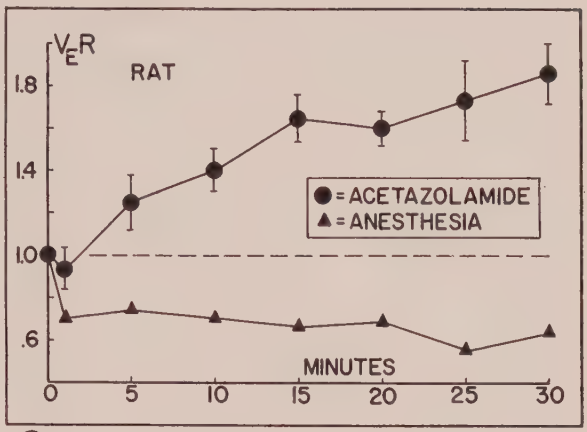




FIG. 1. Simultaneous $CO_2$ and $O_2$ tensions of subcut. pockets after acetazolamide (50 mg/kg I.P.) and CL 13,850. Times after inj. are shown. Lines radiating from points represent stand. error of mean. These lines are drawn on only 2 sides of each point to simplify the illustration.

FIG. 2. Effect of acetazolamide (50 mg/kg I.P.) and pentobarbital anesthesia (29 mg/kg I.P.) on pulmonary ventilation of the rat. Ventilation is expressed as ratio of expired minute volume to control value ($V_E R$). Vertical lines represent stand. error of mean.

for 30 minutes after acetazolamide injection, reaching a mean of 8.6 mm Hg above pre-injection value. It then fell, gradually returning to normal by 90 minutes. There was no significant concomitant change in oxygen tension. Injection of the control substance CL 13,850 produced no significant change in gas composition of similar pockets. These results are shown in Fig. 1. The dose of acetazolamide in these experiments resulted in 98% inhibition of carbonic anhydrase activity in blood 30 minutes after injection as tested by the method described above. Thirty minutes after CL 13,850 there was no measurable enzyme inhibition.

B. *Ventilation.* The effect of acetazolamide on pulmonary ventilation is shown in Fig. 2 where volume of expired air is compared to mean of 3 control values and expressed as a ratio ($V_E R$). Following a short-lived depression, there was a progressive rise in ventilation for 30 minutes. Ventilatory frequency did not change significantly, therefore the rise in minute volume represented an even greater increase in alveolar ventilation. Pentobarbital anesthesia alone produced moderate but progressive ventilatory depression as shown in Fig. 2.

C. *Arterial $CO_2$ content.* The $CO_2$ contents of arterial blood in normal rats, in those following CL 13,850, and after acetazolamide are shown in Table I. The value of 56 volumes % $CO_2$ in animals which were not injected is somewhat high as a result of the method of sampling. Acetazolamide injected at pH 10 resulted in statistically significant rise of 4 volumes % in arterial $CO_2$ content. The arterial $CO_2$ content was not changed following CL 13,850. This absence of effect was predictable since CL 13,850 produced no measurable change in carbonic anhydrase activity and since total amount of NaOH given with it at pH 10 was approximately 0.1 meq. The effects of acetazolamide and CL 13,850 on gas tensions of pockets were similar to the 30 minute values in the first series. There was no significant difference in values for hematocrit as measured in the 3 groups of this series.

*Discussion.* If it be assumed that a sub-

TABLE I. Arterial $CO_2$ Content and Hematocrit of Normal Rats and Those 30 Min. after Injection of Acetazolamide and CL 13,850.

| | # | Arterial $CO_2$ vol, % | Hct. |
|---|---|---|---|
| Acetazolamide | 12 | 60.6 ( .4) | 52 (1.6) |
| CL 13,850 | 11 | 57.7 ( .8) | 51 (1.3) |
| Normal | 6 | 56.4 (1.6) | 48 (2.5) |

Numbers in parentheses are stand. errors of mean.

cutaneous gas pocket approaches equilibrium with venous blood(4), then a rise of $CO_2$ tension in the pocket may result from one or a combination of the following independent variables:

1) Depression of alveolar ventilation, 2) Decrease in blood flow, 3) Increase in metabolic $CO_2$ production, 4) Interference in $CO_2$ transport mechanism.

The mechanism of increase in $CO_2$ tension of subcutaneous pockets following inhibition of carbonic anhydrase will be discussed in terms of these 4 variables.

1. Ventilatory depression with resultant rise in arterial $CO_2$ tension would increase $P_{CO_2}$ in venous blood and in gas pocket unless blood flow increased sufficiently to compensate for rise in arterial blood. This mechanism can be safely dismissed since hyperventilation followed acetazolamide, albeit, these animals were anesthetized while those in which the gas pocket composition was studied were not. This difference in experiment design was necessary since measurement of ventilation in unanesthetized rat is technically unsatisfactory. It is improbable that anesthesia belied the effect of acetazolamide in the conscious animal. The rise in arterial $CO_2$ content with hyperventilation is interesting. We observed this response in the dog following carbonic anhydrase inhibition when renal excretion of base is prevented by ureteral ligation. If the venous $P_{CO_2}$ increases following carbonic anhydrase inhibition, there must be a slow approach toward equilibrium between dissolved $CO_2$ and carbonic acid during the time for transport of venous blood from tissues to lungs. The slight increase in bicarbonate which results would not be lost as $P_{CO_2}$ falls during rapid passage of blood through pulmonary capillaries. Neither would the reaction be completely reversed in arterial blood before it reaches the tissues, since time of transport of arterial blood from lungs to tissues is less than circulation time of venous blood from tissues to lung. The net effect is that since volume of venous blood is greater than the arterial, at any time more blood is exposed to high $CO_2$ tension (venous) than to low (arterial). This must result in a gradual increase in bicarbonate in both arterial and venous blood in the absence of carbonic anhydrase activity, unless there is concomitant selective elimination by the kidney. In the rat the rise in arterial $CO_2$ content is consistent with $CO_2$ retention in the presence of disequilibrium between $CO_2$ and carbonic acid and suggests that renal excretion of base had not occurred by 30 minutes.

2. A rise in venous $CO_2$ tension secondary to a decrease in blood flow must be associated with a fall in oxygen tension. Degree of change would depend upon relative shapes of dissociation curves for $CO_2$ and $O_2$ over involved area of pressure change. The available data for rat blood(6) indicate that the observed rise in venous $CO_2$ tension from 48 to 57 mm Hg would be associated with an extreme fall in oxygen tension almost to zero, its precise value being a function of the exchange ratio. No significant change in oxygen tension of the pocket occurred following acetazolamide. This difference in behavior of the 2 gases was apparently not the result of a difference in rates of exchange between blood and pocket since there was no trend toward a rise in oxygen tension during the 90 minute period. It is therefore concluded that elevation of $CO_2$ tension was not the result of a decrease in perfusion.

3. The observed rise in $CO_2$ tension of the pockets cannot be explained as a result of increase in metabolic $CO_2$ production since there would be, of necessity, an associated increase in oxygen uptake with consequent fall in its tension in venous blood and therefore in the pocket. No such change in oxygen tension occurred.

4. A rise in venous $CO_2$ tension resulting from disequilibrium between $CO_2$ and carbonic acid in the erythrocyte is an adequate explanation of the results of these experiments. $CO_2$ carried by venous blood from tissues is normally transported at a tension which is dependent upon rapid equilibration between dissolved $CO_2$ and bicarbonate made possible by activity of carbonic anhydrase. The high tension of $CO_2$ which develops in the pocket and venous blood when enzyme is inhibited can be interpreted as reflecting a

failure in conversion of $CO_2$ to bicarbonate in the erythrocyte, more being therefore transported in the dissolved and carbamino forms at a higher pressure. The consequences of such increase in venous $CO_2$ tension are those which have been observed following carbonic anhydrase inhibition in the dog(1). The higher tension of $CO_2$ in venous blood must result in retention of $CO_2$, the quantity being dependent upon level of pressure and tissue dissociation constants. This equilibration at a higher level of $Pco_2$ can be accomplished by tissues because they are not dependent upon enzymatic acceleration for conversion of $CO_2$ to bicarbonate, since the reaction is not temporally limited by circulation. $CO_2$ is therefore retained until its tissue stores are increased to a new state of equilibrium.

*Summary.* 1. Venous and tissue gas tensions in the rat have been estimated by use of artificially constructed subcutaneous gas pocket. Inhibition of carbonic anhydrase by acetazolamide results in a rise in venous and tissue $CO_2$ tension independent of change of oxygen tension. This effect is associated with hyperventilation and an increase in arterial $CO_2$ content. 2. These findings are interpreted as direct evidence of $CO_2$ retention resulting from interference in $CO_2$ transport by carbonic anhydrase inhibition.

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## Neutrophil Life Cycle with Tritiated Thymidine.* (24190)

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Patterns of neutrophil balance can be brought into sharper focus than heretofore possible by means of high resolution radioautography with tritium-labeled thymidine. A rather precise localization may be achieved with labeled nucleoside because of its specific incorporation into DNA and the short range of tritium beta particles(1,2). The exogenous precursor is apparently available only for a brief period after administration, which is an added advantage. Neutrophilic leukocytes are part of a highly labile renewal system and the kinetics of their development *in vivo* can be determined from temporal changes in degree of labeling and distribution of the various cells in developmental sequence. The present investigations were initiated to establish the neutrophil life cycle in the normal steady state as a basis for evaluation of various perturbations of the neutrophil system including those induced by irradiation.

*Methods.* Thus far, 2 normal dogs (1 male and 1 female beagle, 1.5 and 4 yrs. old respectively) have been studied after intravenous injection of tritiated thymidine in dosage of 100 $\mu$c/kg body weight. The thymidine, labeled on the pyrimidine moiety, was radiochemically pure as judged by chromatographic and spectrophotometric analyses. Specific activity was 390 mc/mmole.† Bone marrow and peripheral blood studies were carried out at frequent intervals during the first 2 days, and daily thereafter for 2 weeks. Marrow samples (0.8 ml) were aspirated from femur or iliac crest into equal volume of heparinized plasma. The procedure was performed under local anesthesia in trained animals. Peripheral blood studies, which in-

* This work was performed under auspices of U. S. Atomic Energy Com.

† Obtained from Schwarz Laboratories, Mount Vernon, N. Y.

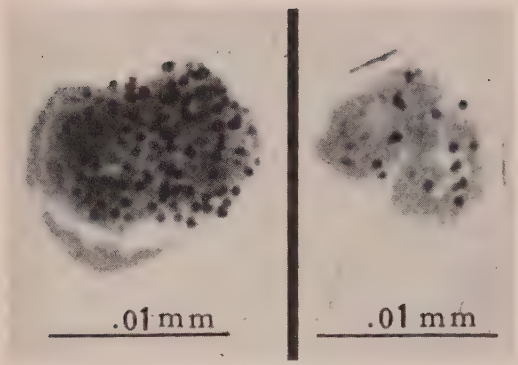


FIG. 1. Left: Radioautograph of myelocyte in marrow 4 hr after inj. of tritiated thymidine. Right: Segmented neutrophil in blood 4 days after inj.

cluded total and differential leukocyte counts, hematocrit, and blood volume, did not reveal any significant changes in neutrophil balance. Thin films of marrow suspension and blood were made on glass slides coated with egg albumin and chrome alum. The preparations were fixed in absolute methyl alcohol and radioautographs were made, using a permeable base stripping emulsion (British Kodak, AR 10)(3). After exposure for 6-8 weeks at 5°C, the preparations were developed, fixed, and treated with Giemsa stain at pH 5.75. Some 300 radioautographs were made for each animal, and, although the various analyses have not been completed, the chronology of neutrophil development *in vivo* is clearly discernible. Counts of labeled cells were based on enumeration of at least 1000 myeloid and associated number of erythroid cells for preliminary analysis of each marrow sample. Since myeloblasts and promyelocytes comprise only a few percent of the total, these cells are included with the myelocytes for the present. In nearly all radioautographs 4 grains over a nuclear area represented a statistically significant increase above background ($p < .01$). Radioautographs of the neutrophil series are shown in Fig. 1.

*Results.* The tritium label appears first in immature cells which ordinarily contribute to renewal of myeloid and erythroid populations by mitosis. About 15% of myelocytes and 20% of erythroblasts in marrow aspirates are labeled within 2 hours after injection of tritiated thymidine in the dosage employed. Labeled lymphocytes are not seen at this time. Densely labeled telophases of both myeloid and erythroid series are found in marrow by 4 hours. When the number of positive cells is computed in terms of normalized distribution of various cell types, there is a rapid increase with time. Since part of the label remains in the daughter cells of a labeled progenitor, several mitoses may be required, depending upon degree of initial incorporation, before the label is no longer detectable above background. The number of positive cells begins to decrease by 48 hours, owing to dilution of the label by mitosis and, possibly, the beginning of release of labeled cells from marrow. Turnover time of proliferating myeloid elements can be approximated from rate of increase of positive cells and is of the order of 15 hours. Consideration of time relationships between thymidine administration and increment in labeled mitoses, as well as the decrement in grain counts should provide a more complete picture of the life cycle of various proliferating cells.

Distribution of labeled myeloid cells progresses in an orderly manner from the less to the more differentiated forms. There is a comparable progression of the label in nucleated erythrocytes. Only 1-3% of lymphocytes are labeled in marrow samples from 12 hours to 5 days after injection of tritiated thymidine. Labeled metamyelocytes appear in bone marrow within 12 to 24 hours and band cells within 24 to 36 hours. Tagged neutrophils (segmented) can be detected in aspirated marrow within 2 to 3 days and in peripheral blood by 3 to 4 days. The first labeled neutrophils may be released from marrow somewhat earlier without detection in circulation owing to their rapid dilution in the large extravascular pool(4). The peak of labeled cells in peripheral blood (25 to 30%) is attained by 4 to 5 days and this is followed by rapid and, perhaps exponential, decrease in percentage of positive cells with a half time of the order of 2 days. Although the peak of labeled neutrophils in blood appears to correspond with that in marrow, there is an unavoidable dilution of marrow with blood

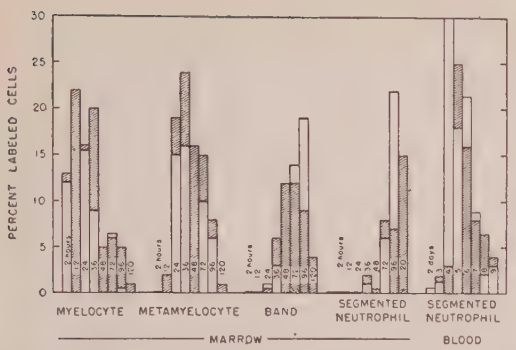


FIG. 2. Time relationship between inj. of tritiated thymidine and appearance of label in the various cells of the neutrophil series. (Hatched bars, male beagle; open bars, female beagle.)

during aspiration, which influences the percentage of labeled segmented neutrophils in marrow. When this factor is considered, it is clear that the true peak in marrow occurs about a day earlier than in the periphery. A similar sequence of segmented neutrophils in marrow and blood has been seen in the mouse with $C^{14}$-labeled adenine(5). It is of interest to note that labeled myelocytes may be detectable in marrow for several days. It can be inferred from this that some labeled neutrophils are released to the circulation for about an equivalent length of time, although in decreasing numbers. This conclusion is supported by an apparent decrease in grain counts of the peripheral neutrophils with time. The overall sequence of events is depicted in Fig. 2. Comparable results were obtained in both dogs.

There is, in general, a reasonable correspondence between these results and the indirect approximations of neutrophil balance reported previously(6,7). It would appear from these data that the time for differentiation of a myelocyte to a segmented neutrophil in the dog is between 2 and 3 days. Another 2 to 3 days are spent in the segmented form. More detailed counting and more frequent sampling is necessary, however, to establish the precise chronology of neutrophil maturation and the life cycle of the proliferating elements.

*Summary.* The pattern of neutrophilic granulocyte development has been studied in 2 dogs by high resolution radioautography with tritiated thymidine. Two to 3 days are required for differentiation from the myelocyte to the segmented neutrophil. The half time for disappearance of the latter in the periphery appears to be of the order of 2 days.

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## Effectiveness of Gluconate, Chloride, and Other Sodium Solutions in Treatment of Experimental Burn Shock.* (24191)

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There is adequate experimental(1,2,3) and clinical(4,5,6,7) evidence for the effectiveness of oral sodium solutions in prevention and treatment of burn shock. For other types of shock the experimental evidence(8,9,10,11) is just as good, but clinical confirmation is meager. There is relatively little evidence on

* Dr. R. Carl Millican and Dr. Sanford M. Rosenthal of N.I.H. made their laboratory available for preliminary experiments. Dr. Stanley Levenson, Walter Reed Army Inst. of Research, furnished encouragement and criticism.

optimum electrolyte composition of solutions for oral use. In particular, there has been no systematic approach to the design of an oral sodium solution which will offer a great improvement in palatability with minimal, if any, loss of effectiveness. Palatability of currently employed solutions has been no great problem in hospital use, but it may be of major importance in treatment of burns *en masse*. Acceptability of iced solution in hospital environment might be expected to be better than acceptability of the same solution in tepid water on battlefield or in temporary facilities of a disaster-stricken city. Our study approached the determination of an effective, but more palatable, oral sodium solution by comparing effectiveness of solutions of a number of sodium salts, singly and in various combinations; and by appraising the influence on effectiveness of various ancillary measures for improving palatability.

*Methods.* Mice were subjected to standard thermal injury, and were treated with various sodium solutions. Mortality measured at 24 hours was chosen as criterion of effectiveness of the solutions, after recommendations of Hamilton *et al.*(12), Rosenthal and Millican (13), and Allen(14). The 24 hour point appears to offer a separation between early deaths, due to shock, and late deaths, due to other factors(15). The anions chosen for study included chloride, because it is the experimental standard; bicarbonate, lactate, citrate, and acetate, because they have been used clinically; gluconate, because it is quite palatable;† and succinate, in spite of its impalatability, because it has been used experimentally with recognition of its influence on respiration of damaged tissue(16,17). Solutions were administered in 2 doses; the first given orally, the second either orally or intraperitoneally. Intraperitoneal injection was selected for the second dose as a matter of convenience. Rosenthal(1) and Millican *et al.*(18) found no difference between the effect of oral and intraperitoneal administration. After preliminary experiments, a total dosage of 18% of body weight was selected. This volume approaches the tolerance of animals for rapid oral administration, and a large dose was desired to increase the probability of detecting any deleterious effect from excessive dosage of anions. Female albino mice were lightly anesthetized with ether and immersed to the axilla in water at 70°C. The hair was not clipped, current practice in Rosenthal's laboratory. The mice were of 2 different strains and weights: 1500 were W. R. Bagg mice of 12-14 g; 1215 were CWL mice of 14-18 g. In individual experiments all mice were of same strain, weight, and nutritional status. Period of immersion was adjusted to strain and weight of animals to obtain in untreated animals a 24 hour mortality of 80% to <100%. All W. R. Bagg mice were immersed for 7 seconds. CWL mice were immersed 8 seconds or 8.5 seconds; 0.5 second was added to immersion time for groups of mice which appeared unusually vigorous and active. In 2 experiments (A, Tables I and II) mortality of untreated animals fell outside the range 80% to <100%. These experiments were repeated (B, Tables I and II). For experiments involving W. R. Bagg mice environmental temperature was 23.5°C ± 1.5°; for CWL mice, 22.8°C ± 0.6°. Immediately after immersion, the first dose was administered by gavage. The second dose followed at 2 hours. With 2 exceptions, all solutions contained sodium as the only cation, and in concentration of 140 meq/l. The Lactated Ringer's Solution was the USP product, and one of the multiple ion formulations contained 145 meq/l of sodium, the additional 5 meq/l being in a flavoring agent. Confidence limits (95%) of percentage mortality were computed by the formula:

$$\text{C.L.} = p \pm \left( t_{(\infty)} \times \sqrt{\frac{pq}{n}} \right).$$

Probability (p) figures for validity of differences in mortality were obtained from standard t tables, with t computed by the formula:

† Dr. Joseph M. White and Miss R. Millard called my attention to the palatability of sodium gluconate.

$$t_x = \frac{p_1 - p_2}{\sqrt{\frac{p_1 q_1}{n_1} + \frac{p_2 q_2}{n_2}}}.$$

All p values quoted are for mathematically independent comparisons of data involving the same immersion times, and animals of same strain, weight, and nutritional status.

*Results. Standard solutions.* Following treatment with sodium chloride, Lactated Ringer's Solution, and the customary(6,7,19) chloride-bicarbonate mixture, mortality was approximately the same (Table III). Mortality following treatment with sodium chloride is much higher than that reported by Millican *et al.*(2) in similar, but not exactly comparable, experiments. "However, the significant criterion is the deviation from control which occurs as a result of treatment . . ." (20).

TABLE I. Mortality following Treatment with Lactate and Chloride as Related to Untreated Mortality.

| Solution* | | | | | | |
|---|---|---|---|---|---|---|
| Chloride | None | 140 | 105 | 70 | 35 | |
| Lactate | None | | 35 | 70 | 105 | 140 |
| Exp. A | | | | | | |
| † | (.70) | (.23) | .33 | .33 | .13 | .33 |
| n | (30) | (30) | 30 | 30 | 30 | 30 |
| Exp. B | | | | | | |
| † | (.97) | (.55) | .73 | .43 | .67 | .77 |
| n | 30 | 30 | 30 | 30 | 30 | 30‡ |

Strain, W. R. Bagg; wt, 12-14 g; immersion, 7 sec.; method of admin., 6% body wt by gavage, 12% intraper.

( ) = Data utilized in Table III.
n = No. of animals.
* Figures = Anions in meq/l.
† Mortality at 24 hr.
‡ Tetany observed.

TABLE III. Mortality following Treatment with Standard Solutions.

| Solution* | None | Cl 140 | Cl 93 $HCO_3$ 47 | Lactated Ringer's |
|---|---|---|---|---|
| † | .84 | .28 | .24 | .31 |
| .95 C.L. | ± .033 | ± .041 | ± .039 | ± .042 |
| n | 120 | 120 | 120 | 120 |

Strain, W. R. Bagg; wt, 12-14 g; immersion, 7 sec.; method of admin., 6% body wt by gavage, 12% intraper.

n = No. of animals.
.95 C. L. = 95% confidence limits.
* Figures = Anions in meq/l.
† Mortality at 24 hr.

*Single anions in concentration of 140 meq/l.* Under our conditions, some anions (lactate, acetate, bicarbonate, citrate) are far less effective than chloride in lowering mortality below that of the control (untreated) group (Table IV). That this lack of effectiveness may be the result of active toxicity is suggested by appearance of tetany in some groups of animals treated with these anions alone or in high concentration with chloride (Tables IV, V). Tetany was never seen in animals treated with gluconate, and only rarely with succinate. The number of animals used in simultaneous comparison, presented in Table IV, is too small to give validity to differences between lactate, acetate, bicarbonate, and citrate, which showed a mortality range of 77-97%. Succinate was significantly more effective than lactate ($p < .02$). In this experiment no valid difference

TABLE II. Mortality following Treatment with Chloride and Gluconate if Injury Is Equal to or Greater Than $LD_{100}$.

| Solution* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Chloride | None | 140 | 122.5 | 105 | 87.5 | 70 | 52.5 | 35 | 17.5 | |
| Gluconate | None | | 17.5 | 35 | 52.5 | 70 | 87.5 | 105 | 122.5 | 140 |
| Exp. A | | | | | | | | | | |
| † | 1.00 | .27 | .47 | .20 | .67 | .60 | .73 | .67 | .93 | .93 |
| n | 30 | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 |
| Exp. B | | | | | | | | | | |
| † | .93 | .13 | .07 | .13 | .07 | .13 | .07 | .07 | .13 | .47 |
| n | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 |

Strain, CWL; wt, 14-18 g; immersion: A, 8.5 sec.; B, 8 sec.; method of admin., 6% body wt by gavage, 12% intraper.

* Figures = Anions in meq/l. † Mortality at 24 hr. n = No. of animals.

TABLE IV. Mortality following Treatment with Various Anions, 140 meq/l.

| Solution | None | Cl | Gluc | Succ | Lact | Acet | $HCO_3$ | Citr | Lactated Ringer's |
|---|---|---|---|---|---|---|---|---|---|
| † | (.93) | (.27) | .40 | .47 | .77 | .77 | .87 | .97 | (.40) |
| n | ( 30) | ( 30) | 30 | 30* | 30* | 30* | 30 | 30 | ( 30) |

Strain, W. R. Bagg; wt, 12-14 g; immersion, 7 sec.; method of admin., 6% of body wt by gavage, 12% intraper. ( ) = Data utilized in Table III.
n = No. of animals.
* Tetany observed. † Mortality at 24 hr.

between gluconate and chloride is demonstrated. However, when total experience with 140 meq/l gluconate solution is compared with experience with 140 meq/l chloride solution, the lesser effectiveness of gluconate is more striking: Cl 23% (n = 90), Gluc 51% (n = 90), no treatment 93% (n = 105); Cl *vs.* Gluc $p<.001$.

*Mixtures of chloride and other anions.* In spite of the relative ineffectiveness of non-chloride anions of sodium when used alone, the results from mixtures of these anions with chloride (Table V) indicate that potency of chloride is generally retained even when it is diluted half and half with a non-chloride anion. The data on gluconate-chloride mixtures (Tables II, V) indicate that even three-quarters of the chloride in a 150 meq/l solution might be replaced with gluconate without pronounced loss of effectiveness.

*Effect of citrate, and free citric acid in mixtures.* It was anticipated that citrate or free citric acid or both might prove deleterious on parenteral injection. Consequently, tests for effectiveness of some formulations containing citrate and citric acid included both oral and intraperitoneal administration. Citrate in concentration of 35 meq/l may be deleterious on intraperitoneal injection, but the mortality data are not conclusive: both doses oral, 20% (n = 30); second dose intraperitoneal, 63% (n = 75). However, these are composite figures from several different experiments. A single paired experiment did not demonstrate a significant difference (oral 40%, intraperitoneal 47%). It is clear that

TABLE V. Composite Mortality following Treatment with Graded Proportions of Chloride and Other Anions.

| Solution | | None | | | | | | | Lactated Ringer's |
|---|---|---|---|---|---|---|---|---|---|
| Chloride* | | | 140 | 105 | 93 | 70 | 35 | | 109 |
| Non-chloride anion* | | | | 35 | 47 | 70 | 105 | 140 | 28 |
| — | | .91 | .36 | | | | | | |
| | | 255 | 270 | | | | | | |
| Gluc | † | | | .23 | | .31 | .30 | .49 | |
| | n | | | 60 | | 75 | 60 | 75 | |
| Succ | † | | | .53 | .50 | .33 | .43 | .55 | |
| | n | | | 60 | 30 | 30 | 30 | 60‡ | |
| Lact | † | | | .53 | .20 | .30 | .31 | .62 | .31 |
| | n | | | 60 | 30 | 90 | 45 | 90‡ | 120 |
| Acet | † | | | .40 | | .50 | .51 | .77 | |
| | n | | | 30 | | 60 | 45 | 30‡ | |
| $HCO_3$ | † | | | .37 | .29 | .43 | .53 | .87 | |
| | n | | | 60 | 90 | 30 | 15‡ | 60‡ | |
| Citr | † | | | .63 | | .60 | .67 | .97 | |
| | n | | | 75 | | 30 | 15‡ | 30 | |

Data not suitable for independent mathematical comparisons. Composite results with W. R. Bagg 13 g mice immersed 7 sec., and CWL 16 g mice immersed 8 and 8.5 sec. Method of admin., 6% body wt by gavage, 12% intraper.
n = No. of animals.
* Figures = Anions in meq/l. † Mortality at 24 hr. ‡ Tetany observed.

TABLE VI. Mortality following Treatment with Solutions Containing Free Citric Acid.

| Solution* | None | Cl 140 | Cl 68, Gluc 46, Citr 26 | | | | | |
|---|---|---|---|---|---|---|---|---|
| Free citric acid, g/l | | 0 | 0 | 1.5 | 3.0 | 3.0 | 4.5 | 4.5 |
| Method of admin. | | P | P | P | P | O | P | O |
| † | .98 | .40 | .47 | .53 | .60 | .27 | .97 | .42 |
| n | 60 | 60 | 15 | 15 | 15 | 15 | 60 | 135 |

Strain, CWL; wt, 14-18 g; immersion, 8.5 sec.
P = 6% of body wt at 0 hr by gavage; 12% of body wt at 2 hr, intraper. O = 9% of body wt at 0 hr by gavage; 9% of body wt at 2 hr by gavage. n = No. of animals.
* Figures = Anions in meq/l. † Mortality at 24 hr.

citrate is tolerated well at 35 meq/l and less on oral administration (Tables VI, VII). Free citric acid, 4.5 g/l, definitely increases mortality when given intraperitoneally (Table VI). Addition of a sweetening agent, and effervescence in the solution, do not appear to impair effectiveness after oral administration (Table VII).

*Discussion.* From a pharmaceutical standpoint, it is very difficult to alter, conceal, or compensate for the taste of chloride in concentrations of 90 meq/l or higher. It would be a great gain in palatability if the chloride component of oral sodium solutions could be reduced below this level. Such appears feasible, from the point of view of burn shock prevention and treatment, with the use of several non-chloride ions (acetate, citrate, lactate, gluconate), which in themselves are quite palatable. Of these, the safest appears to be gluconate, since it is the most effective in 140 meq/l solution, and has not shown a toxic effect under our conditions.

Additional gains in palatability can be obtained by effervescence with $CO_2$, free citric acid, and a sweetening agent.‡ To date, the best compromise I have found between effectiveness in burned mice and subjective palatability to the author's family and neighbors is:§

| | |
|---|---|
| Sodium chloride | 2.0 g |
| " gluconate | 17.4 |
| " bicarbonate | 2.1 |
| Citric acid, anhydrous | 4.8 |
| Sodium cyclohexylsulfamate | 1.0 |

In one liter of water the solution offers a pleasant taste, an attractive fizz, and the following ionic composition:

| | |
|---|---|
| Sodium | 145 meq/l |
| Chloride | 35 |
| Gluconate | 80 |
| Bicarbonate | (converted to citrate) |
| Citrate | 25 |
| Citric acid | 47 |
| Cyclohexylsulfamate | 5 |

This solution is comparable in effectiveness to sodium chloride in prevention and treatment of experimental burn shock in mice under our conditions (Table VII; Cl 42% *vs.* USG-20 39%, p = .7). If used in humans in dosage of 15-18% of body weight, the solution would supply approximately 1000 calories. The proportion of normal caloric requirement supplied to mice was considered negligible (2.5%). A solution containing a larger proportion of chloride (Table VI) might stand on better physiological grounds.

*Conclusions.* 1) Chloride appears to be the most important anion in oral sodium solutions used for prevention and treatment of experimental burn shock in mice. 2) Citrate, ace-

TABLE VII. Mortality following Treatment with Solution Containing Effervescence, Sweetening Agent, and Citric Acid.

| Solution* | None | Cl 140 | Cl 35, Gluc 80, Citr 25‡ | |
|---|---|---|---|---|
| Method of admin. | | P | P | O |
| † | .91 | .42 | .70 | .39 |
| n | 45 | 60 | 30 | 120 |

Strain, CWL; wt, 14-18 g; immersion, 8.5 sec.
P = 6% of body wt at 0 hr by gavage; 12% of body wt at 2 hr, intraper. O = 9% of body wt at 0 hr by gavage; 9% of body wt at 2 hr by gavage. n = No. of animals.
* Figures = Anions in meq/l.
† Mortality at 24 hr.
‡ Plus free citric acid, 3.0 g/l, and sodium cyclohexylsulfamate, 5 meq/l; effervescent — citrate formed by interaction of citric acid and $NaHCO_3$.

‡ All such preparations were prepared through the courtesy of Mr. A. W. Taff, Emerson Drug Co.
§ Formulation USG-20, Emerson Drug Co.

tate, bicarbonate, and lactate are far less effective than chloride, and may be toxic in large doses. 3) There is no sanctity in the customary 93:47 or higher meq ratio between chloride and other ions in oral electrolyte solutions used in these experiments. 4) Gluconate appears to serve as a partial substitute for chloride, and it lends palatability; experimental and clinical studies are indicated on gluconate metabolism in man.

This article is not to be construed as reflecting the position of the U. S. Army or any element thereof. The author alone is responsible for data presented and conclusions drawn.

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## Reversal of Respiratory Decline in Necrotic Liver Degeneration by Intraportal Antioxidants.[1] (24192)

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A specific metabolic lesion, respiratory decline, has been demonstrated in liver slices from rats maintained on a diet producing necrotic liver degeneration(1,2). Normal-appearing slices of such livers are unable to maintain respiration in the Warburg apparatus after initially normal $O_2$ consumption for the first half hour. The defect is characteristic for the latent period of the disease; it precedes the acute pathological lesion by several weeks. Respiratory decline is prevented by feeding of those factors which protect against liver necrosis, *i.e.*, cystine†, Vit. E, and Factor 3. The lesion is reversed within minutes after injection of Vit. E into the portal vein (3)‡, but not after that of Factor 3§. Pre-

[1] This manuscript was originally submitted on April 30, 1956.

* These studies were performed during tenure of Brewer's Yeast Council Research Fellowship.

† Since submission of this paper it has been shown that Factor 3 is an organic selenium compound (Schwarz, K., Foltz, C. M., *J. Am. Chem. Soc.,* 1957, v79, 3292). The protective effect of L-cystine is caused by a trace contamination with Factor 3-active selenium.

‡ *In vitro* addition of α-tocopherol to deficient liver slices, either as an emulsion or in water-soluble forms, has no significant effect on metabolic lesion.

§ Relation of Factor 3-active selenium compounds to respiratory decline will be the subject of a separate report.

viously, evidence was presented for the protective dietary effects of a series of antioxidants, notably of N, N′-diphenyl-p-phenylenediamine (DPPD), against development of necrotic liver degeneration(4). We have investigated the effects of *intraportal injection* of the same series of antioxidants on respiratory decline. In this paper it is shown that certain of these substances cause a reversal of the metabolic lesion identical to that produced by injection of α-tocopherol.

*Methods.* Details concerning methods and materials have been published(4,5). Rats of the Fischer 344 strain, an inbred albino strain, were placed at weaning on the necrogenic, 30% *Torula* yeast diet. The Warburg experiments were usually performed after 15 to 17 days. The average weight of the animals at that time was 42 g. The livers mostly appeared grossly normal; if there was any necrosis, only normal-looking areas were used. *Two step Warburg experiments.* The technic of partial liver extirpation and intraportal injection followed that of Rodnan, *et al.*(3), except that a light ether anesthesia was used instead of Nembutal. In this procedure each animal serves as its own control. The left lateral lobe was removed from the liver, and slices were immediately prepared and incubated. After removal of the left liver lobe, the emulsion containing the antioxidant was injected into the portal vein. Care was taken to avoid hemorrhage. The wound was closed after instillation of approximately 2 cc of saline into the abdominal cavity in order to counteract shock. The animals recovered rapidly from the operation and behaved normally. Thirty minutes later they were sacrificed by decapitation, the rest of the liver was extirpated, and slices were made from the *right* lateral lobe. This lobe was least disturbed by the preceding ligation of the left lobe. Slicing was done on an ice-chilled tray; the slices were blotted on wet filter paper. In each instance, 2 samples were assayed from the pre- and the post-injection lobe. Each Warburg flask contained approximately 90 mg of tissues (± 15 mg), 3 cc of oxygenated Krebs-Ringer phosphate buffer with .01 M glucose, and .2 cc of 30% KOH in the center well. The flask was gassed with oxygen for 30 seconds and, after an initial equilibration period of approximately 4 minutes, incubated at 37.5° for 140 minutes. Readings were taken at 10-minute intervals during the first hour of incubation, and at 20-minute intervals thereafter. *Evaluation.* The results were evaluated by comparing the respiratory decline of the post-injection samples with the corresponding pre-injection controls. Respiratory decline was calculated from the formula: $d = 100 - \frac{Qo_2 \text{ final} \times 100}{Qo_2 \text{ initial}}$. In normal livers, this value for respiratory decline was practically zero. In rats on the necrogenic diet, it ranged from 43% to 85%. From the values for the respiratory decline before and after intraportal injection, reversion (R) of the decline was calculated and expressed in per cent of pre-injection decline: $R = 100 - \frac{dB \times 100}{dA}$, where dA = respiratory decline of initial pre-injection control, dB = respiratory decline of post-injection sample. *Antioxidants.* The compounds used in this investigation have been listed(4). The sequence of presentation is that used in the other report. *Preparation of emulsions.* Ascorbic acid and methylene blue were dissolved in saline. The emulsions of the more water-insoluble antioxidants were prepared from solutions of the substances in minimal amounts of ethanol. These were added to a warm 5% glucose solution containing .25% glyceryl monostearate and blended immediately for 10 minutes in a Potter-Elvehjem homogenizer. For intraportal injections, .2 cc of these emulsions were used. This volume contained .5 mg of glyceryl monostearate, 10 mg glucose, .01 cc or less of ethanol, and the antioxidants in doses ranging from .0125 to 1.0 mg. Each substance was first screened with 2 animals at the 1 mg dose level. If reversion of respiratory decline resulted, 0.2 mg or less were tested. A more detailed dose-response curve was made with DPPD; levels between .0125 and 1 mg were assayed in order to establish an exact comparison with D,L-α-tocopherol.

TABLE I. Effect of Intraportal Antioxidants on Respiratory Decline of Liver Slices.

| Dose in mg | No. of rats | $Q_{O_2}$ (F100)† A Pre-inj. control 0-30 min. | A Pre-inj. control 100-120 min. | B 30-min. post-inj. 0-30 min. | B 30-min. post-inj. 100-120 min. | % respiratory decline‡ dA | % respiratory decline‡ dB | Reversion of respiratory decline§ R |
|---|---|---|---|---|---|---|---|---|
| | | | | None* | | | | |
| — | 4 | 335 | 92 | 344 | 86 | 72 | 75 | 0 (−6 ± 9.3) |
| | | | | Antabuse (tetraethylthiuram disulfide) | | | | |
| .2 | 4 | 328 | 108 | 295 | 95 | 71 | 69 | 0 (−2 ± 9.0) |
| 1.0 | 4 | 346 | 107 | 340 | 230 | 66 | 31 | 55 ± 10 |
| | | | | DBPC (2,6-di-tert-butyl-4-methylphenol) | | | | |
| .2 | 4 | 324 | 152 | 368 | 145 | 53 | 61 | −17 ± 13 |
| 1.0 | 4 | 298 | 69 | 358 | 253 | 73 | 22 | 74 ± 12 |
| | | | | Hydroquinone‖ | | | | |
| 1.0 | 2 | 365 | 207 | 322 | 114 | 44 | 66 | −53 ± 29 |
| | | | | Amylhydroquinone (2,5-di-tert-amylhydroquinone) | | | | |
| .1 | 4 | 292 | 96 | 272 | 91 | 68 | 68.5 | 0 (1 ± 9) |
| .2 | 4 | 332 | 89 | 350 | 299 | 73 | 13 | 84 ± 15 |
| 1.0 | 2 | 318 | 71 | 314 | 308 | 78 | 1 | 97 ± 5 |
| | | | | Santoquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) | | | | |
| .1 | 4 | 338 | 99 | 368 | 178 | 70 | 51 | 27 ± 5 |
| .2 | 4 | 314 | 151 | 351 | 285 | 53 | 18 | 65 ± 14 |
| 1.0 | 4 | 324 | 159 | 328 | 326 | 51 | 3 | 96 ± 11 |
| | | | | Santoflex B (6-phenyl-1,2-dihydro-2,2,4-trimethylquinoline) | | | | |
| .1 | 6 | 280 | 107 | 324 | 273 | 63 | 18 | 71 ± 13 |
| .2 | 4 | 281 | 113 | 293 | 272 | 61 | 7 | 87 ± 4 |
| 1.0 | 4 | 303 | 119 | 306 | 280 | 61 | 8 | 87 ± 5 |
| | | | | DPPD (N,N′-diphenyl-p-phenylenediamine) | | | | |
| .0125 | 6 | 344 | 120 | 337 | 181 | 65 | 46 | 31 ± 9 |
| .01875 | 6 | 314 | 96 | 322 | 270 | 68 | 16 | 75 ± 7 |
| .025 | 7 | 306 | 95 | 303 | 273 | 69 | 10 | 88 ± 8 |
| .05 | 6 | 322 | 61 | 353 | 339 | 81 | 5 | 94 ± 2 |
| .1 | 2 | 324 | 129 | 306 | 293 | 59 | 4 | 92 ± 7 |
| .2 | 6 | 273 | 115 | 320 | 307 | 62 | 3 | 97 ± 7 |
| 1.0 | 2 | 294 | 111 | 303 | 315 | 63 | −4 | 107 ± 7 |
| | | | | D,L-α-tocopherol | | | | |
| .025 | 4 | 297 | 126 | 298 | 158 | 57 | 45 | 19 ± 13 |
| .05 | 6 | 311 | 56 | 335 | 202 | 82 | 41 | 50 ± 5 |
| .075 | 4 | 320 | 88 | 327 | 264 | 73 | 19 | 73 ± 8 |
| .2 | 4 | 315 | 150 | 322 | 310 | 54 | 3 | 94 ± 3 |

* Emulsifying medium without supplement.

† $Q_{O_2}$ (F100) = oxygen consumption (in μl)/hr/100 mg of liver slices; values were calculated for the first 30 min. intervals, and the 100- to 120-min. intervals of incubation (averages, duplicate determinations).

‡ $Q_{O_2}$ (0-30)−$Q_{O_2}$ (100-120), expressed in % of $Q_{O_2}$ (0-30).

§ R = dA minus dB, expressed in % of dA (mean ± stand. error).

‖ Toxic; see text.

*Results.* It is well established that normal liver slices maintain their initial respiratory activity over extended periods of Warburg experimentation‖. Slices from animals during the latent phase of necrotic liver degeneration, by comparison, show a rapid loss of respiratory activity (Table I). After 2 hours of incubation, $O_2$ consumption has on the average declined to ca 30% of the initial 30-minute value. Intraportal injection of the emulsion without supplement neither influenced initial respiration nor respiratory decline of post-injection slices, as compared to pre-injection controls. This indicated that there was no intrinsic difference between 2 lobes of the same

‖ Under prevailing experimental conditions, normal liver slices maintain respiration over 4-6 hrs of incubation at normal or only slightly altered levels.

liver with respect to respiratory decline, and that the emulsifying agents, by themselves, were without influence on the phenomenon¶.

In the dietary studies the compounds used were roughly classified into 3 groups(4). Group I contained substances which may directly or indirectly act as antioxidants, but which are better known for other properties (ascorbic acid, methylene blue, and Antabuse). Some of these showed limited activity when fed. Group II contained antioxidants of phenolic character which are widely used for stabilization of fats and other materials, but which were completely inactive when fed (NDGA, n-Propylgallate, DBPC, BHA, Propylparasept, and also hydroquinone). Group III contained those antioxidants which protected against liver necrosis (di-tert-amylhydroquinone, Santoquin, Santoflex B, and especially DPPD).

In the test against respiratory decline, ascorbic acid and methylene blue were ineffective when injected intraportally at the 1 mg level. Higher doses were not used.** Feeding of high levels of these 2 substances had afforded roughly 30% protection. Antabuse, on the other hand had shown no significant effect in dietary studies, but produced an effect on respiratory decline; approximately 55% reversion was obtained with 1 mg. It is possible that absorption from the gastrointestinal tract may be insufficient in cases where intraportal injection is relatively more effective than feeding.

Five antioxidants of Group II, inactive when fed in the diet were also ineffective after intraportal injection, with the exception of DBPC. The latter produced 74% reversion of respiratory decline when 1 mg was injected. This observation is of interest in connection with the finding by Bunnell, *et al.* that DBPC is comparatively effective in preventing exudative diathesis in chickens(6).

Unsubstituted hydroquinone was toxic; it appeared to enhance respiratory decline. Animals injected with 1 mg hydroquinone recovered poorly, slight convulsions were observed, and consciousness was not completely regained. After the 30-minute interval, the livers showed a deep-red color, with hemorrhagic spots of pin-point size. Conversely, a sterically hindered hydroquinone, di-tert-amylhydroquinone, was found to be quite effective. It reversed respiratory decline practically completely with 1 mg, whereas .2 mg and .1 mg doses caused 84, and 0% reversion, respectively. The effect is in agreement with the activity seen in the dietary assay; the substance prevented liver necrosis when added at .25% to the diet.

The activities of the 3 last substances of Group III are in accord with those of the dietary test: Santoquin at the 1.0, .2, and .1 mg dose level produced 96, 65 and 27% reversion, respectively. It was less active than Santoflex B. Chemically, the two compounds are closely related, Santoflex B containing a phenyl group instead of an ethoxy group. In the dietary assay, Santoflex B had demonstrated an anomaly in that 28% protection had been obtained with .05% in the diet, but dose levels up to .25% had not yielded increased effects. It is evident that this limitation of activity in the dietary assay was eliminated by intraportal application.

Of all antioxidants tested intraportally, DPPD had by far the strongest effect. With doses between .05 and 1 mg, practically 100% reversion of the respiratory lesion was obtained, and 25, 18.75, and 12.5 $\mu$g produced 88, 75 and 31% reversion, respectively. This activity was compared to that of D,L-$\alpha$-tocopherol emulsions assayed concurrently. DPPD was more effective than Vit. E. Approximately 14.5 $\mu$g of DPPD were required for 50% reversion as compared to ca 50 $\mu$g of D,L-$\alpha$-tocopherol for the same effect. On a molar basis, DPPD was 2 times as effective as Vit. E in this test system.

The results obtained with tocopherol are quite comparable to those reported for the

¶ Antioxidants did not influence $O_2$ consumption of normal liver slices, except for hydroquinone which induced a decline.

** Methylene blue changed the liver to a deep blue immediately after intraportal injection, but the color disappeared after a few minutes. After 30 minutes the liver had its normal appearance. In one instance, the dye remained unchanged in one lobe; in this lobe, complete reversion of the respiratory lesion was found.

Sprague-Dawley strain of rats(4), except that the vitamin shows a somewhat higher activity in the Fischer strain. This is in accordance with the fact that for dietary prevention of liver necrosis the Fischer rat requires only half as much Vit. E as the Sprague-Dawley strain. In chickens on a diet producing encephalomalacia, intramuscularly injected DPPD was reported to be approximately equivalent to $\alpha$-tocopherol(6). In our assay system, it is clearly twice as potent as the vitamin.

The results show that the series of 13 antioxidants, tested previously by dietary supplementation, maintained with few exceptions the same relative order of activity when tested against respiratory decline by the intraportal route. It follows that antioxidants which protect against liver necrosis are not simply preventing rancidity of the diet, or autoxidation in the gut; they act within the organism. The effect is not merely an antioxidant effect in the general sense. More specific physical or chemical properties are necessary. On the basis of data on hand, it cannot be decided whether the active compounds ultimately substitute for Vit. E, *e.g.*, in enzyme reactions involving electron transfer, or whether they act through traces of tocopherol in the tissue. The fact that DPPD is twice as active as Vit. E speaks for the first possibility but does not entirely eliminate the second one. The observed effects seem to be analogous to those obtained with methylene blue and other dyes in enzymatic electron transfer systems.

*Summary.* The effects of intraportal injections of antioxidants on respiratory decline in dietary necrotic liver degeneration were investigated by comparing respiration of liver slices from rats before, and 30 minutes after injection. Several antioxidants (DPPD, Santoquin, Santoflex B, and di-tert-amlhydroquinone) were like $\alpha$-tocopherol quite effective in the reversal of respiratory decline at doses of 1 mg or less, while others (NDGA, n-Propylgallate, BHA, Propylparasept, hydroquinone) were without any effect. Screening of 13 different compounds led with but few exceptions to the same scale of relative potencies as that obtained previously for protection against necrotic liver degeneration by feeding of the antioxidants in the necrogenic diet. Intraportal injection of 14.5 $\mu$g of DPPD was required to produce 50% reversal. On a molar basis, DPPD was twice as active as D, L-$\alpha$-tocopherol. It is concluded that the protective effect against necrotic liver degeneration is not due to the prevention of autoxidation in the diet or in the gut; rather, it takes place within the organism itself.

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# Effect of Beta-Sitosterol on Regression of Hypercholesterosis and Atherosclerosis in Chickens.* (24193)

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Many attempts have been made to initiate regression of cholesterol-induced hypercholesterosis and atherosclerosis. Of the substances tested, few were found to be effective (1-6). Beta-sitosterol(7) and di-hydrocholesterol(8) enhanced regression of hypercholesteremia, whereas only beta-sitosterol reduced hepatic cholesterol in the rabbit. Although the chicken undergoes regression of cholesterol-induced atherosclerosis(9,10) few studies have been made to determine whether this process may be facilitated. Beta-sitosterol has been demonstrated to be a most effective agent for preventing cholesterol-induced hypercholesteremia and atherosclerosis in the chicken(11,12). This report is concerned with the effect of beta-sitosterol in diets with and without cholesterol on regression of cholesterol-induced hypercholesterosis and atherosclerosis in the chicken.

*Methods.* Two hundred one-day-old cockerels,† 50 birds/pen, were fed a broiler ration (7% fat, 24% protein, 3% crude fiber) for an 8-week growth period. Feed and water were allowed *ad lib*. At the end of the 8-week growth period, a random sample of birds was bled to determine normal blood cholesterol level. The cockerels were then placed on an atheroma-inducing diet which contained 2% cholesterol in basal diet. The basal diet was composed of broiler ration + 5% cottonseed oil. At the end of 3 weeks, *i.e.*, the atheroma induction period, a random sample of the population was taken to obtain serum, liver, and aorta (aortic arch, thoracic and abdominal aorta), for analysis. The remaining cockerels were then divided into 4 groups and placed upon the following diets for 8 weeks: A, 1% cholesterol in basal diet; B, 1% cholesterol + 4% sitosterol in basal diet; C, 4% sitosterol in basal diet; D, basal diet only. A representative sample of each group was taken after 2 weeks, and remaining birds were sacrificed at the end of 8 weeks. Serum, liver, and aorta were collected, and the chickens were weighed at each sampling. Liver was removed *in toto* from each chicken, placed in a bottle and quick-frozen. For analysis the liver was thawed and homogenized in a Waring Blendor. Blood was drawn by cardiac puncture, placed in a centrifuge tube, allowed to clot, and centrifuged. Serum was collected and quick-frozen. Adventitial tissue was removed from aortas prior to staining with Sudan IV. Plaque areas were visually estimated and graded. After grading, the aortas were lyophilized. Each tissue was extracted with 1:1 ethyl alcohol-acetone. This extract was used for total and free cholesterol, phospholipids, and total lipids determinations. Total and free cholesterol were determined by the method of Sperry and Webb(13). For total lipid determinations, an aliquot of the alcohol-acetone extract was added to chloroform. This mixture was treated as outlined by Folch(14). Total lipids were determined gravimetrically. Lipid phosphorus was estimated by the method of Fiske and Subba-Row on the residue from the total lipid analysis(15).

*Results.* The atheroma-inducing diet elicited the characteristic rise in total and free cholesterol of serum as shown in Table I. Associated with this rise of the cholesterol was an increased lipid content of the serum. During the atheroma regression period total and free cholesterol and total lipids in serum of Group A were maintained at levels attained

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† Smitherman Whites, Smitherman Hatchery, Mooresville, Ind.

TABLE I. Effect of Beta-Sitosterol on Regression of Serum Lipids in the Chicken.

| Time | Group | Total chol.* | Free chol. | F/T | Total lipid |
|---|---|---|---|---|---|
| Control | | 106 ± 2.6† | 24.4 ± 1 | .21 | |
| Induction—3 wk | | 470 ± 47.9 | 146.5 ± 16.3 | .31 | 1030 ± 73 |
| Regression—2 wk | A | 512 ± 78 | 118 ± 19 | .24 | 1269 ± 273 |
| | B | 151 ± 21 | 30 ± 5.2 | .19 | 539 ± 50 |
| | C | 131 ± 14 | 28 ± 4.0 | .21 | 476 ± 32 |
| | D | 176 ± 36 | 45 ± 9.5 | .25 | 547 ± 65 |
| 8 wk | A | 361 ± 53.8 | 94.3 ± 18.9 | .27 | 659 |
| | B | 128.6 ± 5.9 | 20.1 ± 3.9 | .16 | 392 ± 9.5 |
| | C | 123.3 ± 8.8 | 21.5 ± 2.2 | .17 | 382 ± 28 |
| | D | 136.4 ± 11.2 | 24.0 ± 3.0 | .18 | 332 ± 36 |

* Data expressed as mg/100 cc serum. † Stand. error of mean.

during the atheroma induction period. During this same period a highly significant decrease in the lipids discussed above was observed in Groups B, C, and D. This drop in lipids occurred to the greatest extent after only 2 weeks on the regression diet; a further decrease was seen at 8 weeks. Although there was no significant difference between Groups B, C, and D, there was a tendency at 2 and at 8 weeks for serum cholesterol to decrease in the following order: Gp C > Gp B > Gp D. Lipid-phosphorus content of serum was not altered in any significant direction.

Total and free cholesterol and total lipid concentration of the liver in Group A continued to rise during the regression period (Table II). Total and free cholesterol and total lipid concentration of Groups B, C, and D were greatly reduced from the levels found for Group A during this period.

During the regression period, total cholesterol of the liver was significantly lower in Group C than in Groups B and D. At 2 and 8 weeks of the regression period there was no significant difference in total cholesterol between Groups B and D. Groups B and C had significantly lower free cholesterol than had Group D at 2 and 8 weeks, whereas at 8 weeks Group C was significantly lower than Group B. Although the most profound drop in hepatic total cholesterol was observed during the first 2 weeks of the regression period, nevertheless there was a further reduction in hepatic cholesterol of Groups B, C, and D during the 2 to 8 week period. The F/T cholesterol ratio of Groups B, C, and D approached unity with reduction of total hepatic cholesterol. There were no significant differences in total lipids of Groups B, C, and D during the regression period. Liver lipid-phosphorus content did not show any definite trends during any phase of the experiment.

Total and free cholesterol content of the aortas of Group A continued to increase significantly from the atheroma induction period through the regression period (Table III). In Groups B, C, and D total and free cholesterol of the aorta was significantly lower than in Group A at the end of this period.

TABLE II. Effect of Beta-Sitosterol on Regression of Liver Lipids in the Chicken.

| Time | Group | Total chol.* | Free chol. | F/T | Total lipid | P-values for total chol. | |
|---|---|---|---|---|---|---|---|
| Induction—3 wk | | 24.2 ± 1.8† | 8.2 ± .60 | .33 | 69 ± 3.3 | | |
| Regression—2 wk | A | 27.5 ± 1.3 | 8.9 ± .95 | .38 | 72 ± 9.9 | | |
| | B | 6.9 ± 2.2 | 3.6 ± .25 | .66 | 38 ± 2.3 | B–C | .3 |
| | C | 4.4 ± .29 | 3.4 ± .12 | .81 | 37 ± 1.6 | C–D | .05 |
| | D | 6.2 ± .82 | 4.5 ± .68 | .75 | 38 ± 1.7 | D–B | .7 |
| 8 wk | A | 37.4 ± 3.8 | 9.4 ± .33 | .25 | 93 ±15.1 | | |
| | B | 4.2 ± .19 | 3.3 ± .10 | .79 | 40 ± 1.1 | B–C | .01 |
| | C | 3.2 ± .08 | 2.8 ± .06 | .89 | 39 ± 1.2 | C–D | .01 |
| | D | 3.9 ± .09 | 3.5 ± .08 | .90 | 39 ± 2.1 | D–B | .2 |

* Data expressed as mg/g of wet tissue. † Stand. error of mean.

TABLE III. Effect of Beta-Sitosterol on Regression of Aorta Lipids in the Chicken.

| Time | Group | Total chol.* | Free chol. | F/T | Total lipid | P-values for total chol. |
|---|---|---|---|---|---|---|
| Induction—3 wk | | 5.82 ± .32† | 3.73 ± .18 | .65 | 198 ± 10.5 | |
| | | | | | | AI–2 wk AR‡ |
| Regression—2 wk | A | 8.08 ± .92 | 5.34 ± .41 | .69 | 136 ± 17.6 | A P = .05 |
| | B | 7.70 ± .76 | 5.90 ± .39 | .80 | 169 ± 11.2 | B .05 |
| | C | 6.58 ± .51 | 5.29 ± .39 | .81 | 156 ± 12.9 | C .2 |
| | D | 5.69 ± .96 | 4.30 ± .59 | .77 | 179 ± 23.4 | D .9 |
| | | | | | | 2 wk AR–8 wk AR |
| 8 wk | A | 10.81 ± 1.53 | 7.10 ± .64 | .72 | 128 ± 10.3 | A P = .25 |
| | B | 4.84 ± 1.14 | 3.82 ± .68 | .84 | 130 ± 15 | B .05 |
| | C | 3.54 ± .36 | 3.30 ± .41 | .89 | 134 ± 13 | C .01 |
| | D | 3.96 ± .48 | 3.51 ± .44 | .87 | 131 ± 8.9 | D .1 |

* Data expressed as mg/g of freeze-dried tissue. † Stand. error of mean.
‡ AI = atheroma induction; AR = atheroma regression.

The groups receiving cholesterol with and without beta-sitosterol, Groups A and B respectively, manifested an increased total and free cholesterol at 2 weeks of atheroma regression. Group A at 8 weeks had a higher total and free cholesterol than at 2 weeks. Group B, on the contrary, showed a significant decrease in total and free cholesterol from the levels observed at 2 weeks. In the groups receiving no cholesterol with and without sitosterol, Groups C and D respectively, no significant changes of total cholesterol at 2 weeks of atheroma regression were observed. At 8 weeks the total cholesterol was significantly lower than at the 2-week period. Total lipid content of all groups showed a tendency to decrease during the atheroma regression period.

Number and degree of atheromas were not greatly altered at 2 weeks of the regression period, with exception of Group C, which did show a decrease (Table IV). The differences were clearly evident at 8 weeks. During the regression phase of the experiment a very slow rate of plaque reversal was observed in Group A, in spite of an increased cholesterol content of the aorta. This decrease in atheromas may be associated with decreases in total lipids. However, decreases in total lipids occurred to the same degree and rate in Groups B, C, and D, and in these groups atheromas were entirely absent. Total cholesterol of the aorta at 8 weeks also was greatly decreased.

*Discussion.* Cessation of cholesterol feeding with or without sitosterol administration in the chicken resulted in the reduction of hypercholesterosis. Regression also has been observed in other species fed a cholesterol-free diet only(1,2,7,8). Withdrawal of dietary cholesterol results in a negative balance of cholesterol between body tissue and intestinal lumen. The animal body under these conditions may reduce tissue cholesterol by converting cholesterol to bile acids and/or by excreting cholesterol from the intestinal mu-

TABLE IV. Influence of Beta-Sitosterol on Regression of Atheromas in the Chicken.

| Time | Group | No. of birds | % distribution of grades 3 | 2 | 1 | 0 | % aortas w/atheromas |
|---|---|---|---|---|---|---|---|
| Induction—3 wk | | 40 | 3 | 17 | 40 | 40 | 60 |
| Regression—2 wk | A | 16 | 6 | 13 | 31 | 50 | 50 |
| | B | 19 | 5 | 11 | 32 | 52 | 48 |
| | C | 18 | 0 | 6 | 22 | 72 | 28 |
| | D | 16 | 19 | 6 | 19 | 56 | 44 |
| 8 wk | A | 20 | 5 | 5 | 25 | 65 | 35 |
| | B | 20 | 0 | 0 | 5 | 95 | 5 |
| | C | 19 | 0 | 0 | 0 | 100 | 0 |
| | D | 17 | 0 | 0 | 0 | 100 | 0 |

cosa into the intestinal lumen. However, the rate of regression may not be maximal because excreted cholesterol may be reabsorbed. Indeed, when beta-sitosterol was added to a cholesterol-free diet, the degree of regression was slightly increased. This effect was most evident in liver and was paralleled by similar changes in serum and aorta cholesterol concentrations. Hence, it may be assumed that beta-sitosterol in a cholesterol-free diet enhanced the reduction of a cholesterol-induced hypercholesterosis by inhibiting reabsorption of excreted cholesterol.

Peterson(11,12) and Pollak(16) have reported that addition of sitosterol to a cholesterol-containing diet fed to chickens and rabbits prevented hypercholesteremia. In the present study, beta-sitosterol added to a cholesterol-containing diet caused tissue cholesterol regression in the chicken. Rate of reduction was comparable to that of chickens on a cholesterol-free diet. The mode of action of sitosterol presumably is to prevent absorption of cholesterol. Inhibition of cholesterol absorption may be due to 1) formation of relatively insoluble mixed crystals of cholesterol and sitosterol in the intestinal lumen (16,17), 2) inhibition of a cholesterol esterase which presumably is required for cholesterol absorption(18), or 3) competition for acceptor sites on or within the intestinal mucosa(19,20). Thus, when sitosterol is incorporated into a cholesterol-containing diet, the result, in effect, is a cholesterol-free diet.

Regression of cholesterol and atheromatous involvement of the aorta was obtained by a cholesterol-free diet, with and without beta-sitosterol, or by a cholesterol plus beta-sitosterol diet. A regression of atheromas may occur by suspending cholesterol feeding(9,21, 22). All of the treatments mentioned will lower previously elevated blood cholesterol and other lipids. From these observations it may be concluded that atherosclerosis and lipid infiltration of the aorta is dependent upon concentration of lipids in blood. Thus, blood may be assigned the role of a major donor pool of these lipids to the aorta. Lipids of the aorta are not excluded from the general turnover of body lipids. Indeed, experiments with tritium-labeled cholesterol have shown that there is a turnover of aortic cholesterol, albeit slower than in serum and liver (23). The data presented here support this observation in that the liver and serum cholesterol regressed at a greater rate than did that of the aorta.

Recently Curran and Costello(24) have reported that feeding sitosterols to rabbits for 4 weeks led to development of atherosclerotic plaques in rabbits. However, the data of Beher, *et al.*(7,8) do not support this observation. Shipley, *et al.*(25), have recently reported that continuous administration of beta-sitosterol for a period of 2 years did not produce atheromatous involvement in the rat, rabbit, or dog. The data presented here show that beta-sitosterol when fed with or without cholesterol actually resulted in a decreased sterol level of the liver, blood, and aorta. Indeed, when beta-sitosterol was fed alone, sterol concentration in the liver was significantly lower than in the group fed a sterol-free diet, and the sterol content of the serum and aorta was equal to the group fed a cholesterol-free diet.

*Summary and conclusions.* 1. A previously elevated cholesterol concentration in the liver, serum, and aorta of the chicken was reduced by administration of beta-sitosterol in presence or absence of dietary cholesterol, or by withdrawal of dietary cholesterol. 2. Tissue cholesterol concentrations observed as a result of these treatments always approached the normal values. 3. When 4% beta-sitosterol was fed in the diet, total sterol content of liver was slightly lower than in birds fed the basal diet only. Further, total sterol content of the serum and aorta tended to be lower than that of birds fed basal diet only. 4. Atheroma regression in the chicken was accomplished by administration of beta-sitosterol in presence of dietary cholesterol. 5. Atheroma regression in the chicken was paralleled by a drop in cholesterol concentration of the blood and liver.

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## Unilateral Adenectomy in the Cretin Rat.*† (24194)

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Compensatory enlargement of the remaining organ of a paired organ system generally occurs following unilateral removal of the adrenal(1), kidney(2,3), ovary(4), and testis(5). Factors which alter this response to sub-total removal of organ tissues include age, diet(6,7), sex, time between operation and autopsy, castration(8), hypophysectomy(9, 10,11) and thyroidectomy(9). Prior studies have principally involved the use of adult euthyroid rats. No data, however, have come to our attention in which compensatory enlargement of organs was compared in immature hypothyroid (cretin) rats and euthyroid controls of the same age.

*Materials and methods.* Inbred Vanderbilt rats, maintained, *ad libitum,* on Purina Fox Chow (meal) and tap water were used. Cretins were obtained by feeding 0.5% thiouracil§ in Purina Fox Chow to pregnant rats and subsequently to them and their litters from the 14th-16th day of gestation to end of experiment. Controls were maintained, with their mothers, on the same regimen without the thiouracil. Only male control and cretin rats whose body weights at 30 days ranged 100 to 109 g and 29 to 36 g, respectively, and female controls and cretins whose body weights were between 83-88 g and 28-35 g, respectively, were utilized. At 30 days of

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age either the left adrenal, thyroid lobe (including half of the isthmus and imbedded parathyroids), testis, ovary or kidney was removed from cretin and control rats under ether anesthesia. Unilateral removal of non-reproductive organs was performed in both sexes. The ablated organs were cleaned and weighed on a torsion balance to the nearest 0.5 mg and fixed in Orth's fluid. On the 15th post-operative day the animals were killed with ether and the following organs removed and weighed: testes, seminal vesicles, ventral prostate, ovaries, uterus (fluid expressed), liver, kidneys, thyroid, adrenals, heart (void of blood) and hypophysis. Unoperated male and female cretin and control rats were similarly studied at both 30 and 45 days of age. Testes, ovaries, adrenals and kidneys were fixed in Orth's fluid, embedded in paraffin, sectioned at 9 $\mu$ and stained with Harris' hematoxylin and eosin. Pituitary glands were fixed in Zenker-formol solution. Sections (4 $\mu$ thick) of each gland were stained with aldehyde fuchsin according to Halmi's technic(12). Other sections (6 $\mu$ thick) were stained by the periodic-acid Schiff technic. The response of a residual gland was evaluated, not by comparison with the previously excised contralateral gland, but rather, with the non-excised homolateral gland of an intact control or cretin of the same age(13).

*Results.* Pituitaries of intact male and female cretin rats contained rather small, very sparsely scattered gonadotrophs and thyrotrophs, virtually no acidophiles, and revealed no apparent sex difference. On the other hand, intact control rat pituitaries appeared to have attained the glandular appearance described for the adult with fewer gonadotrophs in the hypophyses of female rats. There was an apparent hypertrophy of gonadotrophs in only unilaterally castrated control rats of both sexes. These cells appeared large, pale staining, but not vacuolated. There was no change in number of gonadotrophs. Thyrotrophs, in pituitaries from all cretins of both sexes, appeared as only very early thyroidectomy cells and were not vacuolated.

Adrenal compensatory hypertrophy in cretins of both sexes was only 25% of that observed in control rats (Table I).

In male and female control rats, hemi-thyroidectomy resulted in 48.4% and 43.8% compensatory hypertrophy, respectively, of the remaining thyroid tissue. On the other hand, no significant hypertrophy of the already maximally stimulated residual thyroid lobe was detected in cretins of either sex.

There was no statistical difference in per cent compensatory hypertrophy of the remaining kidney, of either sex, of cretin or control rats as judged by weight (Table I).

Ovarian compensatory hypertrophy was significantly greater in cretin rats (34.4%) than in euthyroid controls (23.1%). Follicular hyperplasia was apparent in ovaries of both thiouracil-fed rats and controls following unilateral castration. No significant difference between uterine weights of hemi-ovariectomized rats (cretin or controls) and their respective intact counterparts was discerned.

Ability of cretins to compensate lost testicular tissue was 5 times greater than that of controls. Despite the dramatic increase in testis weight, no concomitant increase occurred in weights of accessory sex organs of either group.

Although there was pronounced testis hypertrophy in unilaterally orchidectomized cretins, no germinal elements more mature than primary spermatocytes were found. On the other hand, mature spermatozoa were present in both intact and hemi-orchidectomized euthyroid control rats. Hypertrophy of the seminiferous tubules was obvious in residual testes of both control and cretin rats following unilateral castration.

*Discussion.* Cretinism did not appear to produce differential or selective effects upon splanchnic and somatic growth, inasmuch as renal and body weights remained parallel, but impaired. This is in agreement with the constant relation maintained between renal and whole body growth rates of intact rats(6,14). Neither did cretinism affect degree of renal compensatory hypertrophy. On the other hand, responses of certain endocrine glands to unilateral removal, in the cretin, differed markedly from that of the kidney for there was decreased response of adrenals, apparent

TABLE I. Comparison of Extent of Compensatory Hypertrophy of Various Glands 15 Days after Unilateral Adenectomy in 30-Day-Old Cretin Rats with That in Control Rats.

| Unilateral | No. rats ♀ | No. rats ♂ | Body wt (g) at start and end ♀ | | Body wt (g) at start and end ♂ | | RGW‡ (mg/100 g) at autopsy ♀ | RGW‡ (mg/100 g) at autopsy ♂ | % hypertrophy§ ♀ | % hypertrophy§ ♂ |
|---|---|---|---|---|---|---|---|---|---|---|
| Adrenalectomy | | | | | | | | | | |
| *Intact controls | 8 | 8 | 85 | 135 | 105 | 163 | 8.9 ± .1 (12.0) | 6.3 ± .2 (10.4) | | |
| Operated " | 9 | 8 | 87 | 139 | 103 | 183 | 12.9 ± .4 (17.9) | 8.9 ± .1 (16.1) | 44.9 | 41.2 |
| †Intact cretins | 8 | 8 | 30 | 51 | 32 | 51 | 7.8 ± .2 ( 4.0) | 6.9 ± .3 ( 3.4) | | |
| Operated " | 7 | 8 | 31 | 52 | 34 | 60 | 8.8 ± .2 ( 4.6) | 7.6 ± .2 ( 4.5) | 12.8 | 10.7 |
| Thyroidectomy | | | | | | | | | | |
| *Intact controls | | | | | | | 4.1 ± .1 ( 5.5) | 3.3 ± .1 ( 5.5) | | |
| Operated " | 8 | 9 | 83 | 131 | 107 | 163 | 5.9 ± .3 ( 7.2) | 4.9 ± .2 ( 8.2) | 43.8 | 48.4 |
| †Intact cretins | | | | | | | 34.9 ±1.8 (17.8) | 37.4 ±1.6 (19.1) | | |
| Operated " | 7 | 7 | 33 | 54 | 36 | 54 | 35.4 ±2.2 (19.1) | 38.6 ±2.5 (20.7) | 1.4 | 3.2 |
| Nephrectomy | | | | | | | | | | |
| *Intact controls | | | | | | | 446 ± 6 (602) | 462 ± 21 ( 753) | | |
| Operated " | 8 | 8 | 85 | 131 | 104 | 161 | 615 ± 12 (809) | 656 ± 16 (1057) | 37.9 | 42.0 |
| †Intact cretins | | | | | | | 516 ± 15 (263) | 494 ± 9 ( 252) | | |
| Operated " | 7 | 6 | 35 | 53 | 34 | 52 | 733 ± 22 (393) | 691 ± 18 ( 359) | 42.0 | 39.9 |
| Ovariectomy | | | | | | | | | | |
| *Intact controls | | | | | | | 20.4 ± .6 (27.1) | | | |
| Operated " | 9 | | 87 | 133 | | | 25.1 ±1.2 (33.3) | | 23.1 | |
| †Intact cretins | | | | | | | 16.6 ± .9 ( 8.5) | | | |
| Operated " | 8 | | 35 | 57 | | | 22.3 ±1.1 (12.7) | | 34.4 | |
| Orchidectomy | | | | | | | | | | |
| *Intact controls | | | | | | | | 546 ± 15 ( 885) | | |
| Operated " | | 8 | | | 102 | 161 | | 639 ± 9 (1030) | | 17.0 |
| †Intact cretins | | | | | | | | 264 ± 8 ( 136) | | |
| Operated " | | 12 | | | 33 | 50 | | 488 ± 12 ( 244) | | 84.8 |

* Indicates same group of intact controls. † Indicates same group of intact cretins.
‡ RGW = Right glandular wt in mg/100 g body wt. Actual organ wt appears in parentheses.

$$\S\ \%\ \text{hypertrophy} = \frac{\text{RGW (operated)} - \text{RGW (intact)}}{\text{RGW (intact)}} \times 100.$$

absence of compensatory response in the thyroids, and an increased degree of compensatory hypertrophy of ovaries and testes. Maintenance and growth of these latter glands depend largely upon specific trophic hormone stimulation, but no such specific dependence of the kidneys has been demonstrated. On the other hand, the parallelism between renal and body weights might well reflect decreased growth hormone secretion. The well-known paucity of pituitary acidophiles in hypothyroid rats was apparent in the cretins. The catholic influences of growth hormone, however, could scarcely be expected to affect splanchnic and somatic growth selectively.

Thyroid hormone deficiency may(15-18) or may not(9,19) result in adrenal atrophy. Winter and Emery(9) reported that thyroidectomy affected neither size of adrenal nor degree of compensatory hypertrophy in the adult rat. Our data indicate roughly only 12% adrenal compensatory hypertrophy in the cretin, compared with 44% in the normal rat. The virtual absence of pituitary acidophiles in cretin rats might possibly account for the decreased adrenal response.

Absence of thyroid gland compensation in cretin rats may probably be explained as inability of an already maximumly stimulated thyroid to respond to the additional stimulus of hemi-thyroidectomy.

An increase in the number of gonadotrophs in pituitaries of cretin rats following unilateral castration might readily account for the observed increased response of testes and ovaries. However, the essentially chromphobic nature of the pituitary glands of intact cretins mirrored the appearance of pituitaries

from hemi-castrated cretins. It would seem, therefore, that either rapid depletion of gonadotrophin from these cells had occurred or that a slight, but sustained, secretion of hormone stimulated a very sensitive residual gonad. The increased sensitivity of testes and secondary sex organs of thyroidectomized animals(19,20) and of ovaries of thiouracil-fed rats(21,22) to gonadotrophins seems to support the latter possibility.

The various peripheral effects of hypothyroidism upon target-organ responses to pituitary hormones(19-22) must be considered in interpreting our data. On the other hand, the effect may be central, so that the imbalance of the pituitary-thyroid axis in the cretin, induced by thiouracil administration, affects other pituitary-target-organ axes and thereby mechanisms through which compensation normally occurs are either hampered or facilitated.

*Summary.* Degree of compensatory hypertrophy of various glands in the cretin rat was compared with that in euthyroid controls. Per cent renal compensatory hypertrophy in the cretin of either sex was equal to that of the normal animal. Compensatory responses of certain endocrine viscera, however, are dramatically affected. Such responses appear to be greatly lessened in adrenals, virtually abolished in thyroids, and markedly increased in testes and ovaries of cretin rats. Correlations of various glandular responses with pituitary cytology are considered.

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## Latex Agglutination Test for Disseminated Lupus Erythematosus.* (24195)

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Evidence has been presented which suggests that the LE cell factor reacts specifically with materials of nuclear origin. Holman and Kunkel(1) and Friou(2), using immunofluorescent technics, have demonstrated localization of γ globulin from sera of patients with disseminated lupus erythematosus (DLE) onto whole nuclei and nucleoprotein. Robbins *et*

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*al.*(3) found that some DLE sera fixed complement with whole nuclei, nucleoprotein, and desoxyribonucleic acid (DNA). Meischer has reported on agglutination of DNA and nucleoprotein-coated sheep cells by DLE sera (4). Preliminary reports indicate that addition of soluble DNA to some DLE sera induces precipitate formation(3,5). By a modification of the F II latex fixation test(6), polystyrene latex particles could be "coated" with calf thymus nucleoprotein. Latex particles so treated were agglutinated by the majority of DLE sera tested. This report describes our experience with this procedure.

*Materials and methods.* Polystyrene latex particles (diameter size "0.81 $\mu$") were obtained from Dow Chemical Co. An aqueous stock suspension of the particles was prepared as described by Singer and Plotz(6). Glycine-NaOH buffered saline (pH 8.2) served as diluent of all test materials, unless otherwise stated. The constituents were present in the following molarities: NaCl - 0.15 M, Glycine - 0.1 M, NaOH - .0025 M. pH adjustment was made with $Na_2CO_3$. Calf thymus nucleoprotein was prepared by extraction of thymus glands with 1 molar NaCl and reprecipitation 3 times with 0.15 M NaCl(7) and was stored in 1 molar NaCl at 4°C. The nitrogen (Kjeldahl) concentration was 42.2 mg %. Phosphorus concentration(8) was 15 mg %. The nucleoprotein moved as a single component in electrophoretic studies (moving boundary technic) and sedimented as a single component with ultracentrifugation (Spinco model E). Nucleoprotein-latex was prepared by the following method: (a) the 1 molar salt solution of nucleoprotein was diluted 1:6.9 with distilled water, making the salt concentration isotonic, (b) 24 ml of the above were added to 76 ml of buffered saline and centrifuged at 15000 rpm for 30 minutes (International centrifuge, model PR-2 with high speed attachment) (c) the supernatant from above was mixed with 2 ml of the stock latex solution and, without incubation, centrifuged at 15000 rpm for 30 minutes. The supernatant was discarded. The packed latex was dispersed in 100 ml of buffered saline and centrifuged again under same conditions, (d) the washed packed latex was dispersed in 200 ml of buffered saline by vigorous shaking. This suspension constituted the nucleoprotein latex reagent. Plain latex reagent was prepared by washing 2 ml of stock latex solution with 100 ml of buffered saline followed by dispersion in 200 ml of the saline, as above. Sera were obtained from our patients. Patients listed as DLE had clinical syndromes compatible with that disease and at some time had had positive LE cell preparations. Patients with clinical and laboratory features suggestive of DLE but lacking positive LE cell preparations were designated probable DLE. Sera were stored at –20°C. One ml of serum was serially diluted in 1 ml portions of buffered saline in 12 x 75 mm test tubes, the first tube in 1:2 dilution. One ml of nucleoprotein latex reagent was added to each tube and the contents mixed by agitation. The tubes were incubated at 37°C 1 hour and centrifuged at 2000 rpm 10 minutes. Agglutination of latex particles was judged macroscopically with light shaking. The tubes were then incubated overnight in a 37°C water bath, after which they were centrifuged again under same conditions and reinterpreted. Agglutination in a dilution of 1:4 or greater after the second centrifugation was considered positive. All sera showing agglutination of nucleoprotein latex were tested with plain latex reagent. (A few sera, notably sera from patients with rheumatoid arthritis, agglutinated plain latex.) Positive and negative control sera were included in each study. The degree to which various materials could inhibit the agglutinating property of a DLE serum was determined by inhibition studies. Varying amounts of test materials in volumes of 0.5 ml were added to 0.5 ml of a diluted DLE serum. Dilution of serum was such that the ratio of the previously determined agglutination titer to the dilution was 10. (A serum with a nucleoprotein latex titer of 1:128 was diluted 1:12.8.) After incubation at 37°C for 30 minutes, 1 ml of nucleoprotein latex reagent was added and agglutination determined as above. LE cell preparations were made by modification of the technic described by Davis and Eisenstein(9). The criterion for positivity employed was presence of polymorphonuclear leukocytes containing hematoxylin

TABLE I. Sera Which Demonstrated Nucleoprotein Latex Agglutination.

| Diagnosis | Nucleoprotein latex titer* | No. of patients | Pos. LE prep. on same serum |
|---|---|---|---|
| DLE | 4 | 5 | 3 |
| | 8 | 1 | 1 |
| | 16 | 3 | 3 |
| | 32 | 6 | 6 |
| | 64 | 1 | 1 |
| | 128 | 1 | 1 |
| | 256 | 2 | 2 |
| Probable DLE | 4 | 1 | 0 |
| | 64 | 1 | 0 |

* Titers expressed as reciprocals of serum dilutions.

bodies. Rosette formation and nucleophagocytosis were not considered as evidence for a positive test.

*Results.* Sera were obtained from 24 patients with documented DLE, 6 patients with probable DLE, 4 patients with discoid LE, 53 patients with rheumatoid arthritis, and 84 patients with a variety of diagnoses. Table I lists all sera which demonstrated agglutination of nucleoprotein latex but no agglutination of plain latex. (Not listed are sera of 3 patients with rheumatoid arthritis, 1 patient with multiple myeloma, and 1 patient with chronic renal disease, all of which agglutinated both nucleoprotein latex and plain latex reagents.)

Sera which failed to agglutinate nucleoprotein latex are noted in Table II. Inhibition studies were performed with highly polymerized preparation of DNA, ribonucleic acid (RNA) desoxyribose, and histone—all of calf thymus origin.§ (See Materials and methods). These studies are summarized in Table III. Small amounts of DNA were inhibitory, and this inhibition was abolished or diminished by prior incubation of DNA with desoxyribonuclease (DNase). Sodium versenate (EDTA) prevents enzymatic action of DNase by binding divalent cations.

Table IV summarized absorption experiments with 2 DLE sera. Two ml of the test sera were incubated for 12 hours (37°C) with packed latex sediment of 100 ml of both nucleoprotein latex and plain latex reagents. The supernatants after absorption with nucleoprotein latex were negative in LE cell tests. After absorption, serum B.L. no longer agglutinated nucleoprotein latex and the titer of serum J.B. was reduced from 1:32 to 1:2.

*Discussion.* Agglutination of nucleoprotein latex appeared to be specific for DLE. The test correlated closely with the results of LE cell preparations on the same sera. Of particular interest were the consistently negative results with rheumatoid arthritis (RA) sera. Incidence of positive LE cell tests in RA patients remains highly controversial. The divergence of opinion on this point results, at least in part, from the following difficulties: 1. many different technics with varying degrees of sensitivity have been employed for recognition of the LE phenomenon, 2. criteria for positivity are subject to individual interpretation, and 3. with present limitations of clinical identification, confusion of diagnosis between RA and DLE continues. Although we have made no systematic study of the incidence of positive LE cell tests with RA sera, our experience with technics that avoid leukocyte trauma suggests that, if sufficiently rigid criteria are utilized, LE cells are rarely found in diseases other than DLE.

Absorption of DLE sera with nucleoprotein latex removed the agglutination property and the LE cell factor. The present data suggest (1) that the serum factor which induces LE cell formation is the same as that which ag-

TABLE II. Sera Which Failed to Agglutinate Nucleoprotein Latex. (Miscellaneous group includes cases with hepatitis, cirrhosis, myocardial infarction, various neoplasia, gastrointestinal hemorrhage, and chronic renal and pulmonary diseases.)

| Diagnosis | No. of patients | Pos. LE prep. on same serum |
|---|---|---|
| DLE | 5 | 0 |
| Probable DLE | 4 | 0 |
| Discoid LE | 4 | 0 |
| Rheumatoid arthritis | 50 | Not done |
| Degenerative joint disease | 10 | *Idem* |
| Psoriasis and arthritis | 3 | " |
| Acute rheum. fever | 4 | " |
| Penicillin sensitivity | 2 | " |
| Dermatomyositis | 1 | 0 |
| Scleroderma | 1 | 0 |
| Periarteritis | 1 | 0 |
| Miscellaneous | 62 | Not done |

§ Nutritional Biochemicals, Cleveland, Ohio.

TABLE III. Inhibition Studies with DLE Serum (J.B.).

| Test materials | Degree of nucleoprotein latex agglutination — Tube No. 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Inhibiting conc. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| No added material* | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | |
| | Concentration test materials (mg/cc) | | | | | | | | | | |
| | 2.0 | 1.0 | .5 | .25 | .1 | .05 | .02 | .01 | .005 | .002 | |
| DNA | — | 0 | 0 | 0 | 0 | 0 | + | 3+ | 3+ | 3+ | .05 |
| " + DNase† | — | 0 | 0 | + | + | 2+ | 3+ | 3+ | 3+ | 3+ | .5 |
| " + EDTA‡ + DNase | — | 0 | 0 | 0 | 0 | 0 | 2+ | 3+ | 3+ | 3+ | .05 |
| " + EDTA‡ | — | 0 | 0 | 0 | 0 | 0 | + | 3+ | 3+ | 3+ | .05 |
| RNA | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | — | — | — | No inhib. |
| Desoxyribose | 3+ | 3+ | 3+ | 3+ | 3+ | — | — | — | — | — | *Idem* |
| | Concentration histone (mg $N_2$/cc) | | | | | | | | | | |
| | .7 | .28 | .14 | .07 | .02 | .01 | .005 | — | — | — | |
| Histone§ | 4+ | 3+ | 3+ | 3+ | 3+ | 3+ | — | — | — | — | No inhib. |

* Diluted DLE serum, in absence of added materials, demonstrated strong agglutination.
† 2 mg of DNA incubated with .1 mg of DNase for 30 min. (37°C) $MgSO_4$ .003 molar.
‡ Sodium ethylene diamine tetraacetic acid conc. .25 mg/ml. EDTA alone did not influence agglutination.

TABLE IV. Absorption of DLE Sera with Plain Latex and Nucleoprotein Latex (See Text).

| Patient | Serum conditions | Nucleoprotein latex agglutination — Reciprocals of dilution 2 | 4 | 8 | 16 | 32 | LE prep. |
|---|---|---|---|---|---|---|---|
| B.L. | Unabsorbed serum | 3+ | 3+ | 3+ | 2+ | ± | + |
| | Plain latex absorbed serum | 3+ | 3+ | 3+ | 2+ | 0 | + |
| | Nucleoprotein latex absorbed serum | 0 | 0 | 0 | 0 | 0 | 0 |
| J.B. | Unabsorbed serum | 4+ | 3+ | 3+ | 2+ | + | + |
| | Plain latex absorbed serum | 4+ | 3+ | 3+ | 2+ | + | + |
| | Nucleoprotein latex absorbed serum | + | 0 | 0 | 0 | 0 | 0 |

glutinates nucleoprotein latex particles, and (2) that this factor reacts primarily with DNA.

Some properties of the LE cell factor suggest that it may be part of an immunologic reaction with specificity for nuclear constituents. At present, this remains speculative, and there is no evidence that the factor is responsible for the diverse manifestations of DLE.

*Summary.* Polystyrene latex particles, after treatment with calf thymus nucleoprotein, were agglutinated by some DLE sera. The agglutinating property of DLE serum in most cases paralleled the results of LE cell tests. DNA in small amounts inhibited nucleoprotein latex agglutination.

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# A Simplified Method for Determination of Amino Acid Amidases in Tissue Cultures.* (24196)

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Amino acid amidases (AAAases) catalyze enzymic hydrolysis of amino acid amides to corresponding amino acids with concomitant liberation of ammonia(1). For quantitative assay of AAAase activity in tissues either amino acid or ammonia concentration may be measured(1). Development of rapid microcolorimetric technics for determination of ammonia has resulted in numerous procedures which combine microdiffusion with sensitive colorimetric procedures(2,3). The present study was undertaken to incorporate in a compartmented flask the enzymic assay of AAAase from tissues grown *in vitro,* eliminating deproteinization and the transfer of an aliquot prior to microdiffusion. The enzymic-diffusion reactor (EDR) flask is a modification of Seligson and Hirahara's(2) diffusion bottle for measurement of ammonia in blood, erythrocytes, and plasma.

*Materials and methods.* The cortices of bovine kidneys from 14 to 20 inch embryos and swine kidneys from 4-week-old pigs were minced and trypsinized according to the method of Young *et al.*(4). Trypsin-dispersed cells were seeded in T-60 flasks and overlaid with nutrient media containing 20% homologous serum as reported previously(5). After incubation at 37°C in stationary position from 7 to 9 days, the cellular monolayers were harvested and washed twice with Hanks' balanced salt solution. The cells were homogenized in TenBroeck homogenizer at 4°C in 0.02 M phosphate buffer, pH 7.8, containing 0.001 M disodium ethylenediamine tetraacetate to give a final concentration of 5% tissue. This homogenate was stored at −70°C and used within a 5-day period for the assay of AAAase. AAAase activity was measured in compartmented flask shown in Fig. 1. Routinely to a 35 ml EDR flask a mixture of $KHCO_3$ (1 g) and $K_2CO_3 \cdot 1\text{-}\frac{1}{2}\ H_2O$ (0.5 g) was placed in one compartment. Buffer, activating ion, substrate, and homogenate in total volume of 1.2 ml were added to the other compartment. The cell homogenates were diluted so that not more than 0.5 $\mu$M of $NH_3$ was liberated during the incubation period. Control flasks contained similar mixtures with deionized water in place of homogenate or substrate. (Specific quantities are noted in the legends to Table I and Fig. 2.) The ground surface of receiving rods were dipped into 1 N $H_2SO_4$ to the indentation, inserted into the EDR flasks which were incubated at 38°C for 30 or 60 minutes. After incubation the flasks were rotated at RT on a wheel (25 rpm) for 30 minutes. The diffused $NH_3$ trapped by the acid film was washed from the ground surface with 2.5 ml of deionized water. Stone's(6) colorimetric procedure was used for measurement of ammonia. Protein was determined by the method of Lowry *et al.*(7). Specific activity is expressed as $\mu$M of $NH_3$ formed per mg protein per hour. The L-amino acid amides (AAAs) tested were ob-

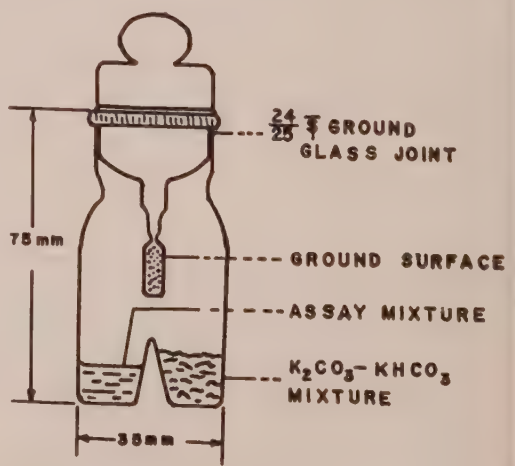


FIG. 1. Enzymic diffusion reactor flask for determination of amino acid amidases.

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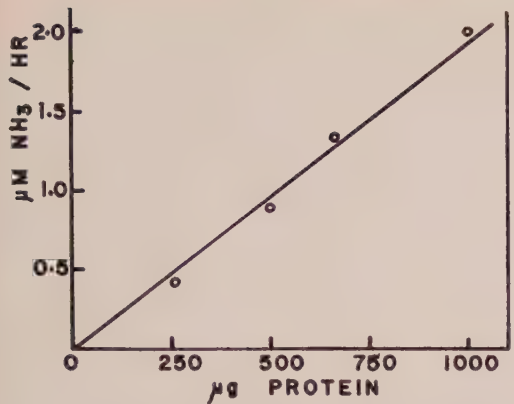


FIG. 2. Effect of enzyme conc. on leucinamidase activity of normal swine kidney cells grown *in vitro*. Assay system contained: L-leucinamide HCl, 100 μM; $MnCl_2$, 30 μM; aliquot of homogenate of the desired protein conc.; and tris buffer, 0.1 M, pH 8.3 to a final vol of 1.2 ml.

tained from the Mann Research Laboratories.

*Results.* Crude cell homogenates of bovine and swine kidney tissue cultures were capable of deamidating L-asparagine, L-glutamine, glycinamide · HCl, L-leucinamide HCl, and L-tyrosine amide. The AAAases from swine kidney tissue had about the same activity as the AAAases from bovine kidney tissue. Asparaginase was found to be the least active and leucinamidase the most active in deamidations of the AAAs tested. Typical data are summarized in Table I.

The activity of leucinamidase as a function of enzyme concentration is shown in Fig. 2. The results show that liberation of $NH_3$ from L-leucinamide HCl was related linearly to the enzyme concentration.

*Discussion.* The results indicate that modification of Seligson and Hiarahara's(2) method provides a rapid, accurate means of measuring enzyme reactions in which $NH_3$ is liberated. Since reproducible values were obtained with crude homogenates the method should be appropriate with purified preparations.

It is recognized by the author that leucine amidase and leucine aminopeptidase could be the same enzyme. Other enzymes may be present in the crude homogenate such as aminopeptidase which could release $NH_3$ if endogenous substrates were present. But, Spackman *et al.*(8) reported that crude enzyme preparations of swine kidney (leucine aminopeptidase) which hydrolyzed L-leucinamide required a prior incubation of 2 to 3 hours with $Mn^{++}$ ions. The author observed almost an immediate effect with $Mn^{++}$ which would tend to indicate the presence of leucinamidase. In this case the estimated AAAase activity, for example leucinamidase, would be large in comparison to the relative rates of the side reactions. The endogenous values were found to be very low and considered beneath the reliable range of the colorimetric procedure. The inclusion of a flask containing the AAA with homogenate omitted, showed negligible non-enzymatic hydrolysis in the assay. In Fig. 2 the rate of $NH_3$ release appears to be proportional to the enzyme concentration over the entire range of concentrations tested. The proportionality suggests that the substrate must be at a sufficiently high concentration to saturate the enzyme throughout the incubation period.

Brown *et al.*(3) have reported that deproteinization of human plasma and rat tissues prior to alkalinization and ammonia diffusion results in a varying degree of amide hydrolysis. Since similar situation has been encountered by the author with cell homogenates the enzyme assay and microdiffusion were combined in a single flask with the omission of a deproteinization procedure. Thus the EDR flask may be used to great advantage in studying enzymic reactions which are dependent upon ammonia determinations.

TABLE I. Deamidation of Amino Acid Amides by Crude Cell Homogenates of Bovine and Swine Kidney Tissue Cultures.

| Substrate | μM $NH_3$/mg protein/hr | |
|---|---|---|
| | Bovine | Swine |
| *L-asparagine | .07 | .05 |
| Glycinamide HCl | .10 | .11 |
| *L-glutamine | .42 | .52 |
| L-tyrosine amide | .82 | 1.08 |
| L-leucinamide HCl | 1.76 | 2.06 |

Assay system for AAAs: L-amino acid, 100 μM; $MnCl_2$, 30 μM; suitable aliquot of homogenate; and tris(hydroxymethyl) aminomethane buffer, 0.1 M, pH 8.3 to final vol of 1.2 ml. The control flasks were similar without L-amino acid amide or homogenate. Incubation period was 30 or 60 min. at 38°C.

Values = Complete System (CS) − [(CS + Substrate) + (CS + Homogenate)].

* $MnCl_2$ was omitted from the assay.

*Summary.* A simple enzymic-diffusion reactor flask is described for the enzymic assay of amino acid amidases from tissues grown *in vitro*. The procedure is rapid, precise, and applicable for measuring enzymic reactions which involve the determination of ammonia.

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## Technics for Separation of Plasma Cholesterol Esters for Determination of Iodine Value, and of Cholesterol.* (24197)

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To investigate the effects of ingestion of such preparations as ethyl linoleate and ethyl oleate upon fatty acid composition of the plasma cholesterol esters, new methodology was required. Such methods are here reported, as well as a modification of the Schoenheimer-Sperry technic for plasma cholesterol determination which substitutes relatively stable colorimetry for the Liebermann-Burchard reaction.

I. *Separation of cholesterol esters from other plasma lipids.* A number of adsorbents were tried in conjunction with various solvents or mixtures of solvents. Activated and non-activated silicic acid was used during the phase of standardization. In our hands, equal parts by weight of celite and "non-activated" silicic acid have produced the best separation. Many investigators using silicic acid for separation of the different lipid components advise certain mesh size and also "activation" of the silicic acid by heat or by using dehydrating solvents. Such procedures increase the efficiency of the column considerably where maximum efficiency is desired, but these additional steps are unnecessary and time-consuming when one is separating small quantities of lipids. Silicic acid, as received from the manufacturer,† contains about 22% water. The column consists of a 25 ml burette with self-lubricating teflon plug valve assembly,‡ and a 125 ml flask fused to the top of the burette as a reservoir. A glass wool plug is introduced, and the celite-silicic acid mixture is added, with tamping, to a height of 10 cm. An aliquot of the plasma filtrate (in acetone alcohol) containing approximately 20 to 30 mg of total lipid is evaporated just to dryness under partial vacuum at approximately 50°C, and the lipids dissolved immediately in a small amount of petroleum ether (5 ml). This is poured upon the *dry* column. (In a recent report, Hirsch also recommends addition of the dissolved lipids to the column in the dry state)(1). The test tube containing the lipids is washed 3 times with 2 ml portions of petroleum ether. These are added to the column and allowed to flow in without pressure until the liquid has been completely absorbed. The cholesterol esters are eluted with a total volume of 100 ml of petroleum ether under slight positive pressure to maintain a flow rate of approximately 1 ml per

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† J. T. Baker Chemical Co., Phillipsburg, N. J.

‡ "Ultramax", Fischer and Porter Co.

minute. Efficiency of the separation is checked by comparing values for free and total cholesterol determined on the eluate with those obtained by direct analysis of the original acetone-alcohol filtrate and with values for total fat determined by Bragdon's method (2) on the eluate. Values for cholesterol in a series of 29 unselected eluates differed from direct determination for esterified cholesterol on the same filtrates by an average of 7.3 ± 5.1§ mg %, which was 4.8 ± 3.7% of the direct values. No free cholesterol was present in the eluates, nor were other lipid fractions eluted with the cholesterol esters. This last fact was confirmed by the good agreement shown between values for total fat determined directly on aliquots of eluates and total fat values calculated from the ester cholesterol using the factors given by Bragdon(2) for cholesterol esterified with a $C_{18}$ fatty acid. In the same series of 29 samples as above, the mean difference between total fat determined directly and calculated was 9.00 ± 3.00§ mg % or 6.2 ± 1.12%§ of the direct value. The cholesterol esters constitute approximately 35% of the total lipids in normal plasma. However, in some patients with xanthomatosis, in spite of the marked absolute increase in cholesterol, the cholesterol esters may represent only 10%, the bulk of the lipid being neutral fat. Satisfactory separation has been obtained even in such plasma.

II. *Colorimetric determination of the iodine value of fats.* The pyridine sulfate dibromide reagent of Rosenmund-Kuhnhenn as modified by Yasuda(3) has been approved by Bloor (4) for determination of the iodine value of blood lipids. Using this procedure, the theoretical iodine value is obtained for cholesterol and a number of other blood lipids. Conversion of this titrimetric procedure to a simple colorimetric method enables one to use smaller amounts of material for analysis and increases sensitivity as well as accuracy of the technic. A known amount of pyridine sulfate dibromide is added to the lipid which has been dissolved in chloroform. As the fatty acids are saturated with bromine, the yellow color disappears. This extinction is measured colorimetrically. Oleic acid in amounts ranging from 1 to 6 mg is likewise treated with the reagent. The standard curve thus prepared is used to calculate iodine value of the lipid, using 89.9 for the iodine number of oleic acid.

*Reagents.* 1. 0.10 N pyridine sulfate dibromide. Measure 16.5 ml (16 g) of purified pyridine and 10.9 ml (20 g) of concentrated sulfuric acid into separate flasks each containing about 40 ml glacial acetic acid. Add these reagents slowly, with cooling, then combine. Into a third flask containing 40 ml glacial acetic acid, add 5 ml (16 g) bromine. Add this to the first mixture and bring the entire volume to 2000 ml with glacial acetic acid. Store in a dark bottle. This reagent is good for one year. The working solution should be prepared daily by adding 3 parts (by volume) of glacial acetic acid to 1 part of the stock pyridine sulfate dibromide reagent. 2. Chloroform (reagent grade). 3. Glacial acetic acid.

*Procedure.* Dissolve fat to be analyzed (containing preferably between 2 and 4 mg lipids) in 5 ml chloroform. Add 3 ml working dibromide reagent, mix, filter rapidly through No. 40 Whatman filter paper into test tubes. Cap tightly with parafilm and place in the dark. (Avoid contact with parafilm). At the same time prepare standards and 2 blanks, one containing 5 ml chloroform and 3 ml pyridine sulfate-dibromide solution and a second blank containing 5 ml chloroform and 3 ml glacial acetic acid. Read after 30 minutes in a photoelectric colorimeter using No. 42 (blue) filter. Set the colorimeter to the zero mark with the chloroform-acetic acid blank. Then read the reagent blank, standards, and the unknowns. Do not expose the tubes to sunlight or to fluorescent light. Fig. 1 shows a typical standard curve for oleic acid.

*Calculations.* If the quantity of fat used for iodine value determination is not known, an aliquot should be taken for total fat analysis. Bragdon's method yields satisfactory results. Example: Fat content of sample

§ Standard error of mean.

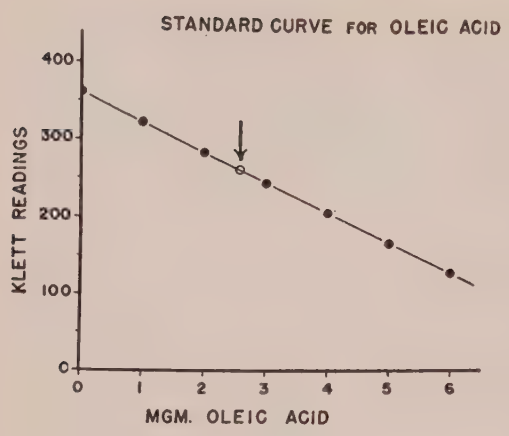


FIG. 1.

3.50 mg, Klett reading 260 = 2.58 mg as oleic acid from the curve, $\frac{2.58}{3.50} \times 89.9 = 66.3$ iodine number of fat,

$$\frac{\text{Reading from curve as mg of oleic acid}}{\text{Total fat}} \times 89.9 = \text{iodine number of unknown.}$$

Using this simple color extinction procedure we have been able to obtain the theoretical iodine value for cholesterol and the reproducibility in duplicate analysis is excellent compared with the more difficult iodine titration using starch as the indicator. In a series of 50 samples, the average variation between duplicate analyses was ± 0.4%.

III. *Modified Schoenheimer-Sperry cholesterol method.* The cholesterol method here reported differs from that described by Schoenheimer and Sperry(5) chiefly in regard to the color development and the solvent wash. In the Schoenheimer and Sperry method, after precipitation and washing of the cholesterol as the digitonide, the Liebermann-Burchard reaction is used, with development of a transient color. This reaction occurs only with unsaturated sterols. Orcinol reacts with digitonin rather than with unsaturated sterols and yields a color which is stable for more than 2 hours (see below). In development of the Schoenheimer-Sperry technic, the micro-colorimetric method was shown to be in agreement with the gravimetric cholesterol digitonide method of Windaus. The present method yields values which agree well with determinations by the Schoenheimer-Sperry method. Representative comparative determinations are shown in Fig. 2. In a series of 12 determinations by both the Schoenheimer-Sperry and orcinol methods, the mean difference was 5.8 ± 1.12§ mg % or 2.6 ± 0.49%§ of the Schoenheimer-Sperry values for total cholesterol, and 4.7 ± 0.88 mg %§ or 7.4 ± 1.51%§ for free cholesterol.

*Reagents.* 1. *Orcinol.* 3 g orcinol is added to 1.00 liter of glacial acetic acid (reagent grade). Then 0.4 ml superoxol is added and mixed. This reagent is good for many months but should stand for 4 weeks before being used. 2. *Isopropyl wash.* Add 50 ml water to 450 ml of 99% isopropyl alcohol. A pinch of cholesterol digitonide is then added, and the solution is shaken well. The amount used should be filtered before use. 3. *Ferric chloride.* Add 1 ml of 10% ferric chloride to approximately 500 ml distilled water, then add 500 ml concentrated hydrochloric acid (reagent grade) and mix.

*Color development using orcinol reagent.* After precipitation of the cholesterol with digitonin,‖ centrifugation and pouring of the solvent containing the excess digitonin, the tubes are inverted and placed on clean filter paper for complete drainage. The precipitate and the sides of the tube are then washed with 3 ml of the isopropyl wash. The tubes are

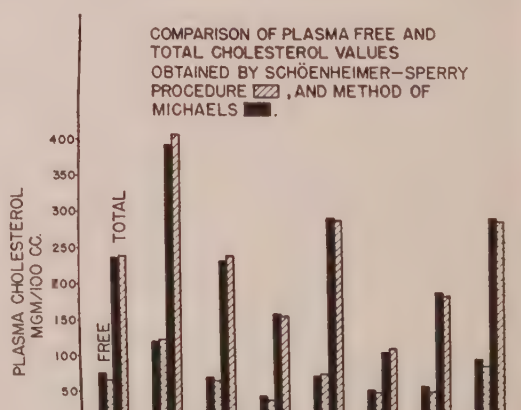


FIG. 2.

‖ Reagent Digitonin, Merck and Co., Inc., Rahway, N. J.

again centrifuged, decanted, inverted, and allowed to drain for at least 5 minutes. To the washed digitonide precipitate add 5 ml of the orcinol reagent and 2 ml of the acid ferric chloride reagent. Cap the top of the tube with parafilm and mix thoroughly. It is best to set up at least 2 standards and run them through the entire procedure. We use a stock standard containing 2.50 mg of cholesterol per ml, and a working standard containing 0.10 mg of cholesterol. Both of these standards are made up in acetone-alcohol and are stable indefinitely. Two blanks are prepared, one containing orcinol and ferric chloride, and the second, in addition, containing digitonin (1 ml of a 1% solution). The latter is included to check for completeness of removal of excess digitonin by the isopropyl wash. The two blanks should yield identical readings, within the limits of accuracy of the colorimeter. All the tubes are heated in a boiling water bath for 20 minutes and cooled. It is necessary for the water bath to be evenly heated throughout. Differing temperatures in different sections of the water bath will give poor results. The colorimetric readings are made at 540 mμ wave length. Some change in color will occur if the tubes are left in direct sunlight; otherwise, no demonstrable change occurs if the readings are made within 2 hours after cooling. The number of duplicate determinations of free and total cholesterol carried out in this laboratory by this method exceeds ten thousand. Duplicates vary by less than ± 3%.

*Summary.* 1. A method for quantitative chromatographic separation of cholesterol esters from other blood lipids is presented. 2. A colorimetric micromethod for determination of the iodine number of plasma lipid is described. 3. A more stable reagent and a more stable color reaction than the Liebermann-Burchard reaction for the determination of plasma cholesterol is presented. 4. Employment of the above methods, together with determination of total lipids by the method of Bragdon, permits determinations of net unsaturation of the fatty acids esterified with cholesterol in the plasma.

Grateful acknowledgement is made to Merck, Sharp and Dohme for generous supplies of Digitonin.

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## Plasma Cholesterol Ester Fatty Acid Composition in Relation to Diet.* (24198)

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In our previous paper it was noted that in a young normal adult male subject during the intake of 80 g of ethyl linoleate daily, the level of plasma cholesterol fell in a remarkable fashion and the iodine value of the cholesterol ester fatty acids at times exceeded 250(1). The present report includes data dealing with fatty acid composition of the plasma cholesterol esters in 4 male and one female subjects during the course of controlled studies.

*Methods.* Cholesterol esters were fractionated by column chromatography as described by Michaels, *et al.*(2) and unsaturated fatty acids determined by the Michaels modi-

* These studies were supported in part by grants from Alameda Co. Heart Assn., The Carnation Co. and Nat. Inst. of Health.

TABLE I. Patient EMAR.

| | | Plasma total lipids | Total cholesterol | Cholesterol ester fatty acids | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Saturated | Mono-enoic | Di-enoic | Tri-enoic | Tetra-enoic |
| Diet: | 10/28 | 553 | 195 | 18.4 | 38.5 | 35.2 | 3.2 | 3.6 |
| Coconut oil, 60 g | /31 | 562 | 175 | 30.8 | 22.5 | 38.8 | 2.4 | 4.0 |
| Protein, 104 g | 11/ 4 | 522 | 150 | 28.6 | 17.7 | 43.7 | 3.6 | 5.0 |
| Carbohydrate, 300 g | / 7 | 500 | 157 | 9.3 | 47.6 | 34.8 | 2.7 | 4.3 |
| | /11 | 603 | 180 | 16.6 | 60.4 | 20.2 | 2.7 | 3.6 |
| | /14 | 572 | 163 | 0 | 67.3 | 23.8 | 3.3 | 4.1 |
| Diet: | 11/18 | 537 | 165 | 0 | 72.5 | 21.1 | 3.1 | 2.3 |
| Ethyl oleate, 60 g | /21 | 475 | 128 | 0 | 68.5 | 20.9 | 4.1 | 5.0 |
| Protein, 104 g | /25 | 447 | 116 | 0 | 74.5 | 19.1 | 3.1 | 1.7 |
| Carbohydrate, 300 g | /27 | 472 | 120 | 0 | 0 | 0 | 0 | 0 |
| | /29 | 441 | 118 | 0 | 72.7 | 19.4 | 4.3 | 2.5 |
| | 12/ 2 | 481 | 135 | 0 | 84.6 | 9.2 | 2.3 | 2.3 |
| | / 4 | 503 | 133 | 5.9 | 68.5 | 13.9 | 3.6 | 3.7 |
| | / 6 | 503 | 128 | 0 | 67.1 | 16.5 | 4.4 | 4.4 |
| Diet: | 12/ 9 | 522 | 134 | 0 | 72.9 | 19.8 | 4.6 | 1.8 |
| Ethyl linoleate, 60 g | /11 | 453 | 123 | 0 | 51.3 | 40.8 | 3.2 | 3.2 |
| Protein, 104 g | /13 | 462 | 136 | 0 | 47.6 | 45.1 | 2.0 | 3.5 |
| Carbohydrate, 300 g | /16 | 453 | 126 | 0 | 36.6 | 55.4 | 3.1 | 3.4 |
| | /18 | 391 | 103 | 0 | 6.5 | 82.5 | 2.9 | 5.9 |
| | /20 | 412 | 110 | 0 | 30.5 | 58.4 | 5.2 | 4.2 |
| | /23 | 412 | 118 | 11.2 | 6.7 | 63.3 | 3.8 | 4.3 |
| | /25 | 428 | 111 | 0 | 0 | 0 | 0 | 0 |
| | /27 | 387 | 105 | 15.9 | 0 | 69.4 | 5.2 | 11.6 |
| | /30 | 384 | 105 | 11.6 | 0 | 68.6 | 8.2 | 7.8 |

fication of the method of Riemenschneider(3). Total lipids were determined by the method of Bragdon(4). Plasma cholesterol was determined by a modification of the method of Schoenheimer and Sperry(2).

*Observations.* The first patient, EMAR, a 61-year-old male with osteoporosis, but with no obvious manifestations of vascular disease, was placed on a chemically constant formula diet, which, for periods of 3 weeks each, contained coconut oil, ethyl oleate, and ethyl linoleate, respectively. The ethyl oleate was 99.8% pure; the ethyl linoleate was more than 95.0% pure. The remainder of the diet was constituted as shown in Table I. All of these diets were thoroughly fortified with essential vitamins and minerals and with crude liver. Protein was derived from non-fat milk solids. Prior to institution of the study, the patient had been on a diet containing relatively large amounts of saturated fat.

During the period on coconut oil, total cholesterol averaged 170 mg % and total lipids 552 mg %. During the period on ethyl oleate, total cholesterol averaged 130 mg% and total lipids averaged 457 mg %. During the period on ethyl linoleate, total cholesterol averaged 117 mg % and total lipids 430 mg %. The final values on linoleate were 105 and 384 respectively.

During the period on ethyl oleate, the mono-enoic acid in the cholesterol ester approached 80% and the di-enoic acid approximated 20%. The ratio was approximately reversed during ingestion of ethyl linoleate.

Within the limits of experimental error, the contents of penta-enoic and hexa-enoic acids did not vary during this study, and were in each instance less than 5% of the total. Except for the final portion of the study, the tri- and tetra-enoic acids were 5% or less.

Stool sterol excretion at all times was less than one gram daily, and did not appear to vary predictably in relation to the kind of fat which was fed.

Patient HMEN, aged 55, a diabetic with evidence of considerable vascular disease, received during a period of 32 days, ethyl linoleate in amounts varying from 50 to 100 g daily, and, for 28 days, 100 g daily of ethyl oleate. The composition of the diet is shown in Fig. 1. As in the previous patient, the mono-enoic acid content of the esters was high during oleate ingestion, and the di-enoic

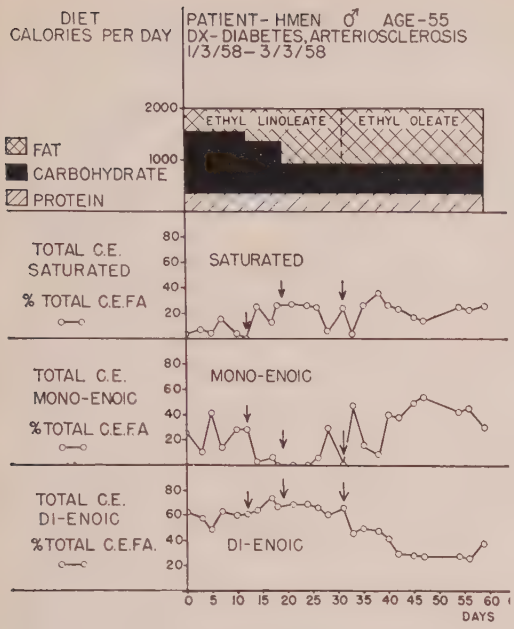


FIG. 1. Cholesterol ester fatty acids in patient HMEN.

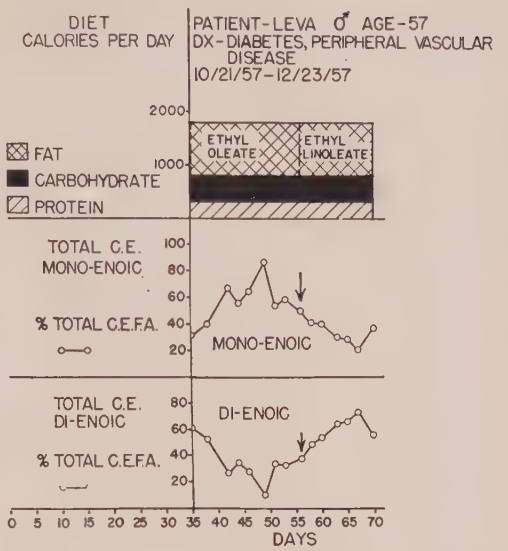


FIG. 2. Effects of oleate and linoleate on cholesterol ester fatty acid composition in patient LEVA.

content was low. The opposite of this was observed during the linoleate intake.

Content of tri-enoic, tetra-enoic, penta-enoic and hexa-enoic acids were low throughout, and, within the limits of error of the method, bore no obvious relationship to the type of fat that was fed.

Patient LEVA, a 57-year-old male mild diabetic, with extensive vascular disease, as in the case of the 2 preceding patients had a relatively high mono-enoic acid and low di-enoic acid content of the cholesterol esters during oleate ingestion and the opposite pattern during linoleate ingestion (Fig. 2). Tri-, tetra-, penta-, and hexa-enoic acid content was low, and, within the error of the method, bore no relationship to the fat which was fed.

The fourth patient, EROS, a middle-aged female, received a totally fat-free, iso-caloric diet. She, like the other patients, was in nitrogen equilibrium or positive balance throughout the study.

In Table II, it is apparent that the mono- and di-enoic acid content of her cholesterol esters followed the same pattern as that noted during oleate administration in the previous patients. Tetra-enoic acids averaged more than 5% of the total cholesterol ester fatty acids.

The fifth patient, AMOR, aged 47, differed from the other diabetics in that his diabetes was severe and totally insulin-dependent. During a 3-week portion of the study he received all of his fats in the form of coconut oil. During the remaining 4 weeks, he received fat mixtures containing the amounts of saturated fats, oleic acid and linoleic acid, respectively, shown in Table III. The progressive hypercholesteremia and hyperlipidemia noted during the coconut oil ingestion is apparent, and the return of lipid values toward normal during the intake of fat containing appreciable amounts of linoleic acid is equally apparent. The stool sterol excretion did not vary significantly during intake of the different fats, nor did the total stool fats. The patient was maintained in weight and nitrogen equilibrium throughout the study.

In the case of the mono-enoic and di-enoic fatty acids, the response pattern is essentially identical with the 3 patients described above. During the period of coconut oil ingestion, tetra-enoic acid content of the cholesterol esters averaged in excess of 5% of total fatty acids.

TABLE II. Patient EROS.

| | | Plasma total lipids | Total cholesterol | Cholesterol ester fatty acids | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Saturated | Mono-enoic | Di-enoic | Tri-enoic | Tetra-enoic |
| Diet: | 1/ 3 | 878 | 256 | 22 | 0 | 65 | 3.9 | 5.6 |
| Protein, 97 g | / 6 | 1016 | 210 | 41 | 0 | 44 | 5.5 | 5.6 |
| Carbohydrate, 300 g | / 9 | 1372 | 228 | 10.5 | 46 | 30 | 3.8 | 5.0 |
| | /13 | 1453 | 260 | 4.5 | 60 | 25 | 3.3 | 5.4 |
| | /16 | 1484 | 263 | 40 | 26 | 24 | 3.3 | 5.9 |
| | /20 | 1316 | 248 | 5 | 63 | 21 | 3.8 | 5.4 |
| | /23 | 1275 | 235 | 20 | 46 | 24 | 4.8 | 5.2 |
| | /27 | 1103 | 235 | 18 | 52 | 22 | 2.4 | 4.5 |
| | /30 | 1062 | 198 | 25 | 40 | 22 | 4.4 | 4.9 |
| | 2/ 3 | 1031 | 196 | 33 | 54 | 19.5 | 4.4 | 5.5 |

In this patient, as in most but not all patients studied to date, the saturated fatty acid content of the cholesterol esters was highest during intake of saturated fat.

*Discussion.* Only 2 consistent observations emerge from these studies: (1) Plasma cholesterol esters mirror to a significant degree the kind of fat which is fed under the experimental conditions used, and in the type of patient studied. (2) Fall in plasma cholesterol and total lipids observed in all individuals during intake of relatively large amounts of lineoleic acid was associated with a relatively high level of di-enoic acid in the cholesterol ester.

In one patient who received a high carbohydrate, fat-free diet, the cholesterol ester composition was much the same as in the patients who received oleate. A much higher plasma total lipid level was observed in this patient than in patients receiving oleate.

In none of these patients, even during high linoleate intake, were cholesterol ester fatty acid iodine values in excess of 180 noted, (as opposed to values of 250 in a young normal male, previously reported). Subsequent studies in normal young adults may help to establish the nature of the polyunsaturated fatty acids responsible for this high iodine value.

*Summary.* Administration of large amounts of purified linoleic acid preparations

TABLE III. Patient AMOR.

| | | Plasma total lipids | Total cholesterol | Cholesterol ester fatty acids | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Saturated | Mono-enoic | Di-enoic | Tri-enoic | Tetra-enoic |
| Dietary fat: | 9/30 | 844 | 205 | 4.6 | 71.5 | 21.8 | .5 | 1.3 |
| Coconut oil, 100% | 10/ 2 | 934 | 229 | 43.2 | 16.7 | 34.1 | .8 | 4.1 |
| | / 3 | 906 | 230 | 40.7 | 15.7 | 33.0 | 3.0 | 5.1 |
| | / 7 | 1119 | 269 | 51.7 | .0 | 34.9 | 4.5 | 6.5 |
| | / 9 | 1412 | 305 | 31.4 | 31.3 | 25.0 | 4.8 | 5.1 |
| | /10 | 1178 | 325 | | | | | |
| | /14 | 1394 | 395 | 17.1 | 49.2 | 26.5 | 2.0 | 4.5 |
| | /17 | 1250 | 365 | 9.6 | 48.5 | 29.6 | .6 | 6.1 |
| Dietary fat: | 10/21 | 1400 | 395 | 7.3 | 57.5 | 26.5 | 3.1 | 4.8 |
| Linoleic, 22.6% | /24 | 1562 | 431 | .98 | 50.2 | 36.9 | 1.8 | 3.8 |
| Oleic, 27.4% | /28 | 1394 | 407 | 0 | 57.1 | 44.8 | 1.9 | 3.4 |
| Saturated, 45% | | | | | | | | |
| Dietary fat:* | 10/30 | 1500 | 350 | 5.2 | .0 | 81.4 | 4.7 | 6.2 |
| Linoleic, 45% | /31 | 1428 | 375 | 15.8 | 10.7 | 64.7 | 2.7 | 4.8 |
| Oleic, 20% | 11/ 4 | 1212 | 312 | .0 | 21.1 | 74.8 | 1.2 | 4.0 |
| Saturated, 29% | / 7 | 1353 | 343 | 3.8 | 2.1 | 82.6 | 4.9 | 4.4 |
| | /11 | 1084 | 285 | 5.9 | 17.2 | 70.0 | 1.7 | 3.3 |
| | /14 | 1037 | 269 | 5.5 | 10.2 | 77.5 | 1.9 | 3.3 |
| | /18 | 1103 | 250 | .0 | 21.8 | 69.8 | 1.7 | 3.0 |

* "Swirl" (Procter and Gamble).

to diabetic and non-diabetic patients results in a marked increase in the di-enoic acid content of the cholesterol esters, in association with a lowering in the mono-enoic acid content. When ethyl esters of oleic acid are substituted for the ethyl linoleate the opposite pattern is observed. A high carbohydrate, fat-free diet produces much the same pattern of cholesterol ester composition as that seen during oleate ingestion.

Grateful acknowledgement is made to Procter and Gamble Research Labs. for supplies of purified oleate and linoleate.

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## Ketosis in the Rat Fetus.* (24199)

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Although placental transfer of many substances has been demonstrated(1-3), such studies of ketone bodies have not been made. Since ketosis may readily develop in the pregnant animal when deprived of insulin(4,5) or when food intake is limited(5-7), it seemed of interest to determine if the ketone body level in fetal blood was related to that in maternal blood.

*Methods.* Sprague-Dawley rats pregnant 17 to 20 days and fed Purina Chow were used. Five of these rats were "totally" pancreatectomized(8) after 17-hour fast and tested 23 hours later. They were not fed postoperatively. Controls consisted of pregnant normal rats fasted 17 and 40 hours. Maternal blood samples for glucose determinations were taken from the tail. Immediately thereafter, mother rats were anesthetized with ether. Fluid was drawn from amniotic cavity with a hypodermic needle and syringe. Fetal blood samples were obtained by removing the fetus from the uterus, cutting open the chest and heart, and collecting the blood on a spot plate with heparin. Generally a single fetus was used for each determination. After obtaining fetal blood samples, blood for determination of maternal levels of ketone bodies and fat was drawn from the aorta with a syringe and needle. Glucose was measured by the method of Haslewood and Strookman(9), ketone bodies by the method of Bessman(10) and total lipids by the method of Bragdon(11).

*Results.* There was a marked elevation of glucose, ketone bodies, and fat in blood of the pancreatectomized rats (Fig. 1). Blood ketone bodies of the fasted normal pregnant rats were also increased, whereas their glucose levels were less than those in the fed rats. There was no significant difference in blood ketone body levels between pancreatectomized and 40-hour fasted normal pregnant rats. Preliminary observations indicate that the marked ketosis observed in the fasting pregnant rat does not occur before 14th day of gestation. Blood fats of some of the fasted normal pregnant rats were also increased.

In agreement with observations of others (1,2), maternal and fetal blood glucose levels were essentially the same. In the diabetics maternal glucose levels (260-420 mg/100 ml) averaged 60 mg/100 ml higher than fetal levels. These data reflect the known rapid transfer of glucose across the placenta(2). Glucose levels in the amniotic fluid of both normals and diabetics were 20 to 30 mg/100 ml lower than those in the fetal blood.

Maternal blood ketone body levels ranged from 0.6 to 114 mg/100 ml (Fig. 1). Over

* The excellent technical assistance of Barbara Guarco and Theresa R. Clary is gratefully acknowledged.

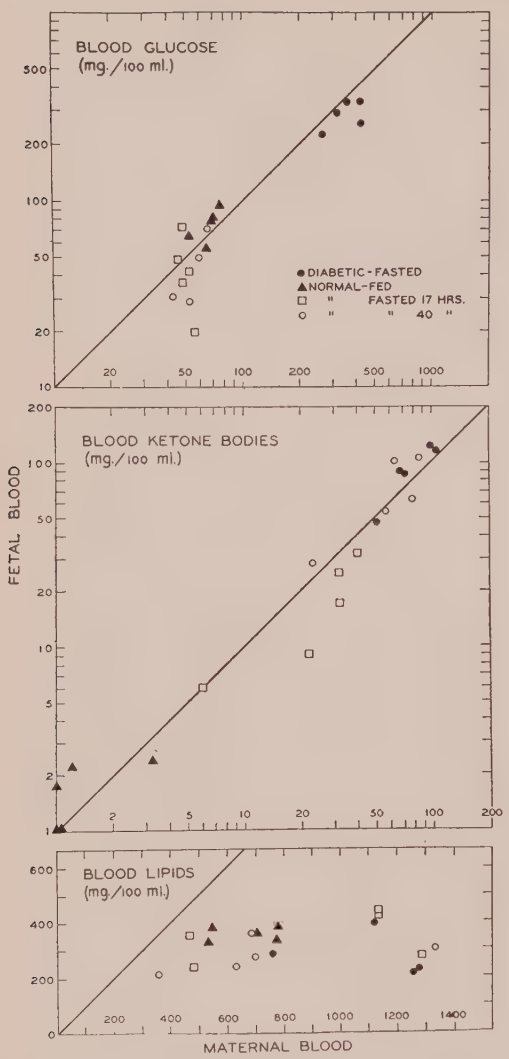


FIG. 1. Comparison of levels of glucose, ketone bodies (as acetone) and total lipids (as palmitic acid) in fetal blood with those in maternal blood. The diagonal line at 45° angle represents the theoretical distribution of observations if there were no differences between fetal and maternal blood.

this wide range, blood ketone body concentration of the fetus equalled that of its mother. The very close agreement of ketone body levels in fetal and maternal blood strongly suggests that ketone bodies are rapidly transferred across the placenta. The ketone body concentration in amniotic fluid also paralleled that in maternal blood.

It has been demonstrated that fats cross the placenta very slowly(1,3). In agreement with these observations, fetal blood lipids were found to be independent of maternal levels (Fig. 1). There were no detectable amounts of fat in the amniotic fluid.

*Discussion.* The cause of increased severity of fasting ketosis during the last third of pregnancy is not known(5-7). Since blood glucose is not elevated and glucose administration readily corrects the ketotic state(7), the ketosis cannot be attributed to insulin deficiency as in the diabetic. MacKay and Barnes have shown that the adrenal gland is necessary for ketosis in fasting pregnant rats (6). Others have observed that plasma level of 17-hydroxycorticosteroids and urinary excretion of glycogenic corticoids are markedly increased during the last third of pregnancy (12-14). It is well-known that cortisone is antiketogenic by virtue of its gluconeogenic action(15,16). However, in severe insulin deficiency, in which carbohydrate utilization is impaired, cortisone is strongly ketogenic(16). If the rate of gluconeogenesis in the fasting pregnant animal is inadequate, a severe carbohydrate deficiency may develop in maternal tissues due to high priority of the fetus for nutrients(17). In such a case it is very likely that the elevated level of corticoids in the mother would result in severe ketosis. The effects of ketosis in the fetus are not known.

*Summary.* Blood levels of ketone bodies, glucose, and fats in pregnant rats were altered by fasting and by pancreatectomy. The concentration of ketones and glucose in fetal blood changed with maternal level, whereas the lipids were independent of maternal level. Ketone bodies appear to cross the placenta as rapidly as glucose.

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## Fat Deposition in Rat Tibia Due to Lysine Deficiency.* (24200)

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Recent publications(1-5) have shown that amino acid composition of the diet may have a decided effect on bone structure. Bavetta *et al.*(1,2) described pathological changes in bones and teeth of rats fed diets deficient in lysine or tryptophan. Specific effects should also be evident in the chemical composition of bone. Harris *et al.*(6) previously observed an abnormal, fatty bone marrow in histological sections from rats fed lysine-deficient gliadin. Quantitative changes in fat content of the tibiae of rats fed diets containing various amounts of lysine are described in this report.

*Methods.* Rats reared on stock diet(7) were weaned at 21 days with average weight of about 50 g. They were caged individually and fed the experimental diets, using litter mate distribution with 3 males and 3 females for each diet. At termination of the 5-week feeding period the animals in each group were weighed, killed, and their tibiae analyzed. For this purpose, the tibiae were dissected from tissues of right hind legs. After cleaning, the raw weight for the pooled group of 6 tibiae was obtained. All analyses were calculated on the basis of this weight. For moisture determination the bones were dried at 100°C. For fat determination the bones were then boiled in 95% alcohol under reflux for 24 hours and extracted with ether in a Soxhlet for same period of time. The bones were dried again and fat content calculated by difference. Finally the dried fat-free bones were ashed at 800°C. Having established amount of water, fat and ash present, the remaining constituents of raw bone have been called "organic residue" by Chick *et al.* (8). The basal diet used consisted of 90% commercial bread, dried at room temperature, supplemented with vitamins, minerals and fat. The bread contained 4% non-fat milk solids. This diet is low in lysine and was previously used by Rosenberg and Rohdenburg(7) to investigate improvement in growth and protein efficiency attainable by supplementation with lysine. An additional group of rats was fed the stock diet to study normal bone composition.

The results of adding increasing amounts of L-lysine monohydrochloride† to the bread diet are summarized in Table I. Fat content of tibiae of rats fed the unsupplemented bread diet was greater than that observed when stock diet was fed. Addition of lysine to the bread diet improved both growth and bone composition. With increasing lysine supplementation there was a decrease in fat content

* Our attention was originally called to this problem by Dr. Theodore F. Zucker of Columbia University, to whom we wish to express our appreciation.

† Du Pont L-lysine monohydrochloride, containing 95% L-lysine monohydrochloride and 5% D-lysine monohydrochloride.

TABLE I. Effect of Lysine on Rat Growth and Composition of Tibiae.

| Diet | % L-lysine in diet | 5-wk gain (g) | Pooled tibiae analysis (6 rats) Raw tibia wt (g) | % water | % fat extracted | % bone ash | % organic residue | % protein (N × 6.25) |
|---|---|---|---|---|---|---|---|---|
| Control bread | .29 | 84 | 1.7600 | 38.03 | 4.39 | 36.59 | 20.99 | 19.31 |
| Bread + .2% L-lysine monohydrochloride | .45 | 143 | 2.1852 | 38.59 | 3.12 | 36.88 | 21.41 | 20.68 |
| Bread + .5% L-lysine monohydrochloride | .69 | 186 | 2.5693 | 39.50 | 2.39 | 36.59 | 21.52 | 21.31 |
| Bread + 1.0% L-lysine monohydrochloride | 1.09 | 187 | 2.5566 | 39.11 | 2.70 | 36.20 | 21.99 | 20.12 |
| Stock diet | 1.10 | 204 | 2.7750 | 40.06 | 2.32 | 36.09 | 21.53 | 19.87 |

of the tibia. The per cent of bone ash and organic residue was constant with all 5 diets, but amount of moisture varied inversely with fat content. Thus lysine deficiency produced a fatty marrow with low moisture in the bones.

Protein content of the raw bones of each group was determined by Kjeldahl analysis of the tibiae from the left hind legs. It is evident that the organic residue is composed almost exclusively of protein.

These observations concerning changes in lipid content of tibiae of rats fed bread diets have been confirmed in experiments with other diets of low lysine content. Details of these experiments, as well as observations on the fat content of the tibiae of normal animals over their life-span, will be published separately.

*Summary.* Results with lysine-supplemented experimental diets and with stock diet show that the lipid content of the tibia is relatively low when sufficient lysine for optimum growth is available to young rats. With the lysine-deficient diet, growth is retarded and bone lipids are increased. Interference with production of normal bone marrow appears to be a definite indication of lysine deficiency.

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## An *in vitro* Study of Fatty Acid Absorption.*† (24201)

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The form in which lipids are absorbed has been the subject of a number of investigations (1). Evidence has been accumulated in support of each of the 2 major theories of fat absorption, partition or lipolytic. The 2 theories have in common the necessity of free

* The author gratefully acknowledges the suggestions of Profs. H. C. Tidwell and T. Hastings Wilson during this investigation and technical assistance of Joanne Keeling and George Nunn.

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fatty acid production but to different degrees. However, the actual mechanism by which the fatty acids are absorbed is still not clear. One of the difficulties in interpretation of previous results is the absence of an *in vitro* procedure in which it is possible to measure precisely the material absorbed. A modification of an existing *in vitro* technic, employed in carbohydrate absorption(2), has been utilized in this study of absorption of $C^{14}$-labeled palmitic acid.

*Methods and materials. Preparation of fatty acid-albumin complex.* The complex was prepared by a modification of a method by Felts(3) in which 20 mg of palmitic acid and 0.7 $\mu$c of $C^{14}$ palmitic acid were dissolved in ether and mixed with 4 ml of ethanol. The mixture was neutralized with 1.8 ml of 0.1 N NaOH and taken to dryness under nitrogen. Ten ml of a 10% albumin‡ solution in bicarbonate-saline, pH 7.0(4), was added and the mixture shaken in mechanical shaker for 1 hour. The solution was then filtered through a sintered glass filter, the pH adjusted to 7.4, glucose added to make a final concentration of 200 mg percent, and the volume made to 15 ml with albumin solution. *Preparation of tissue.* Segments of small intestine of male golden hamster were washed and everted according to procedure described by Wilson and Wiseman(2). In preparation of the sacs, a modification of the existing method was employed by introducing a small polyethylene tubing at one end during preparation of the everted sac, in order to fill the sac with the serosal solution. The tubing was stoppered with a small glass rod. By removing the stopper and introducing a small needle, the serosal solution could be removed without contamination. Sacs for aerobic and anaerobic experiments were prepared from the same animal. The sacs were filled with approximately 1 ml of bicarbonate buffer, (pH 7.4), containing 200 mg % glucose and were incubated 2 hr at 37°C in 3.5 ml of the $C^{14}$ palmitic acid-albumin complex in an atmosphere of either 95% oxygen-5% $CO_2$ or 95% nitrogen-5% $CO_2$. At completion of incubation period, aliquots of serosal and mucosal solutions, and homogenized intestinal wall were plated for counting. The remainder of these samples were combined and fractionated into glycerides and fatty acids. *Fractionation methods.* Mucosal and serosal solutions and intestinal wall were extracted and fractionated into fatty acids and glycerides, with the aid of a carrier, according to the method of Borgström(5), at completion of incubation.

*Results.* After incubation under aerobic conditions, approximately 5% of the original activity, initially placed on the mucosal side, was found in the serosal compartment. When the pooled serosal solutions were fractionated into fatty acids and glycerides, approximately 90% of the serosal activity was found in glyceride form and 10% as free fatty acid. The intestinal wall contained approximately 20% of the original activity distributed as 60% glyceride and 40% free fatty acid. The mucosal solution at completion of experiment, contained 65% of original activity. Fractionation of the mucosal solution into glyceride and fatty acid gave values of 10% and 90% respectively. When a sample of the original palmitic acid-albumin complex, which had not been exposed to the intestine, was subjected to an identical fractionation, average values of 99% fatty acid and 1% glycerides were obtained.

In contrast to above results, total activity on the serosal side, after incubation under

TABLE I. Total Activity Recovered from Mucosal and Serosal Solutions and Intestinal Wall after Placing $C^{14}$-Palmitic Acid-Albumin Complex on Mucosal Side of the Isolated Intestine.

| Incubation atmosphere* | Total activity after incubation based on initial activity (%) | | |
|---|---|---|---|
| | Mucosal side | Intestinal wall | Serosal side |
| Aerobic | 63.3 | 19.4 | 7.2 |
| " | 62.9 | 22.6 | 4.8 |
| " | 67.2 | 21.4 | 4.6 |
| Avg | 64.5 | 21.1 | 5.5 |
| Anaerobic | 90.3 | 1.0 | .4 |
| " | 90.6 | 1.4 | .2 |
| " | 89.7 | 1.4 | .15 |
| Avg | 90.2 | 1.3 | .25 |

‡ Bovine albumin powder (Fraction V), Armour Labs., Kankakee, Ill.

* Aerobic and anaerobic atmosphere 95% $O_2$–5% $CO_2$ and 95% $N_2$–5% $CO_2$, respectively.

anaerobic conditions was on the order of 0.2%. Activity of this fraction was too low for fractionation. The intestinal wall and mucosal solutions gave average values of 1.3% and 90.2% of the total activity, respectively. The resulting mucosal solution after fractionation gave a distribution of activity between fatty acid and glyceride similar to that of the original solution. The intestinal wall under these conditions contained only a small fraction of activity as compared to that found under aerobic conditions. This trace of activity was distributed as 90% glyceride and 10% fatty acid.

*Discussion.* With this procedure a fatty acid, a product produced during digestion of fats, has been shown to penetrate the intestinal wall, be converted into a glyceride, and be transported into the serosal solution. The resulting adsorbed material found on the serosal side was in the form of a glyceride, which is the major product appearing in lymph, when either fatty acids or the esterified fatty acids are fed to the intact animal. An investigation to determine the nature of this glyceride is presently underway.

Evidence has been presented to indicate that the transport from mucosal to serosal side is an energy requiring process, since this transport is decreased approximately 20 fold under anaerobic conditions. It has been possible during the process of absorption and transport to demonstrate the incorporation, which in itself is an energy requiring reaction, of fatty acid into a glyceride. This evidence is substantiated by analysis of the intestinal wall at completion of the experiment. Actual uptake of activity by the intestine, under anaerobic conditions, is decreased 16-fold as compared to aerobic conditions. In addition to decreased energy supply under anaerobic conditions, a factor which may contribute to the lower levels of transformation of the fatty acid into glyceride is a stripping of intestinal mucosa under anaerobic conditions. However, this loss of some of the mucosa does not account for the decrease in activity of the intestinal wall glycerides, since the glyceride in the mucosal solution is not increased over control value.

The increasing percentage of glyceride in the direction, mucosal to intestinal wall to serosal compartment, is paralleled by a decreasing percentage of fatty acid. This finding is consistent with the postulate of entrance of fatty acid into the mucosal cell, transformation into glyceride form, and restricted passage of the glyceride into the serosal solution. The 10% glyceride found on the mucosal side is presently under investigation. This phenomenon may be involved in (a) secretion of glycerides into the intestine(6, 7), (b) reported mechanism of transesterification(8), and (c) controlling factor for rate of fat absorption.

*Summary.* An *in vitro* procedure has been adapted for study of fatty acid absorption. Free palmitic acid enters the intestinal mucosa and is transformed into a glyceride, which is subsequently transferred to the serosal side. The resulting activity on the serosal side is present as 90% or more in the form of glycerides. Transfer of activity to the serosal side and incorporation of activity in the intestine, is inhibited 20- and 16-fold, respectively, under anaerobic conditions. The relationships of the reported findings to fatty acid absorption are discussed.

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# Purification of Follicle-Stimulating Hormone from Human Pituitary Glands.* (24202)

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Highly purified follicle-stimulating (FSH) and interstitial-cell stimulating (ICSH) hormones have been prepared from sheep and hog pituitaries(1-3). Although it is known that human pituitary glands contain both FSH and ICSH activities(4), no purification of these two hormones from extracts of human pituitaries has been reported. In this paper is presented a procedure for the preparation of an FSH concentrate from human pituitaries.

*Material and methods.* Human pituitaries† were obtained at autopsy, one to 3 days after death; the whole pituitary was immediately frozen and lyophilized. The dried pituitary was cut into small pieces and ground before extraction. The assay method of Steelman and Pohley(5), based on augmentation of ovarian weight elicited by FSH and human chorionic gonadotropin (HCG) in intact rats, was used for estimating follicle-stimulating potency of the preparations. Immature female rats of the Long-Evans strain, 21 and 22 days of age, were injected, once daily for 3 days, intraperitoneally with HCG and subcutaneously with the FSH preparation to be tested. The dose of HCG was adjusted to give control ovaries weighing 30-35 mg. Autopsy was performed 24 hours after last injection. Biological activity of a preparation was given as a slope unit defined by the equation:

$$\text{Slope unit} = \frac{\text{Test ovaries (mg)} - \text{Control ovaries (mg)}}{\text{Total dose (mg)}}$$

The HCG preparation was a commercial product‡ and had an activity of 1000 I.U./mg. The preparations of both HCG and FSH were dissolved in distilled water for injection.

*Purification procedure.* All steps were performed at 0-2°C with the exception of the step involving ion exchange resin, conducted at room temperature. *$Ca(OH)_2$ Extraction and $(NH_4)_2SO_4$ Fractionation.* 10 g of human pituitary powder are stirred vigorously with 400 ml of $Ca(OH)_2$ solution at pH 10.3 for about 3 hours. The suspension is set aside for 16 hours and centrifuged; the residue is washed with 100 ml $Ca(OH)_2$ solution of pH 10.3. The supernatant fluid and washing are combined and brought to 1.9 M with respect to ammonium sulfate by addition of solid $(NH_4)_2SO_4$. The precipitate formed is centrifuged off and saved for isolation of growth hormone(6,7). The 1.9 M $(NH_4)_2SO_4$ supernatant fluid is brought to 3 M by addition of more solid $(NH_4)_2SO_4$ at pH 7, and the precipitate which forms is dissolved in water, dialyzed and lyophilized. The lyophilized product (*Fraction SI*) weighs 0.5 g and has an activity of 144 slope units. *Refractionation with $(NH_4)_2SO_4$.* 0.5 g of Fraction SI is dissolved in 50 ml distilled water; after removal of some insoluble material by centrifugation, the supernatant fluid is made to 1.9 M $(NH_4)_2SO_4$ by addition of saturated $(NH_4)_2SO_4$ solution at pH 7. If precipitation occurs, the solution is centrifuged and the precipitate discarded. The $(NH_4)_2SO_4$ concentration in supernatant fluid is increased to 2.7 M by further addition of saturated $(NH_4)_2SO_4$ solution. The precipitate which forms is dissolved, dialyzed, and lyophilized. The lyophilized production (*Fraction SII*) weighs 0.25 g and has an activity of 190 slope units. *Purification on Cation Exchange Resin.* Further purification of Fraction SII is effected on the carboxyl cation exchange resin Amberlite IRC-50 (XE-

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† The author wishes to thank Drs. H. D. Moon and R. Escamilla at Univ. of California Med. Center, for efforts in securing these glands.

‡ The author wishes to thank Leo Pharmaceutical Products, Copenhagen for generous supply of HCG.

TABLE I. Protocol for Purification of Human FSH

| Fraction | Wt, g | Procedure | Bioassay: Total dose, mg | Bioassay: No. of rats | Bioassay: Ovarian wt: HCG alone, mg | Bioassay: Ovarian wt: Test fraction + HCG, mg | Activity, slope units |
|---|---|---|---|---|---|---|---|
| A | 10 | Lyophilized whole pituitaries | | | | | |
| AS | 4.9 | Extracted with pH 10.5 CaO solution | 1.0 | 8 | 33.2 | 63.5 | 30 |
| SI | .5 | 1.9-3.0 M $(NH_4)_2SO_4$ precipitate | .2 | 13 | 37.6 | 66.5 | 144 |
| SII | .25 | Refractionation with $(NH_4)_2SO_4$ | .2 | 13 | 32.4 | 70.4 | 190 |
| CU | .14 | Unadsorbed fraction from IRC-50 column | .1 | 21 | 32.9 | 67.5 | 346 |
| F | .10 | $(NH_4)_2SO_4$ fractionation as SII | .05 | 15 | 37.4 | 60.6 | 464 |

97). 250 mg of Fraction SII are dissolved in 50 ml of pH 5.1 buffer§ and applied to the resin column‖ (0.9 cm diameter column containing 50 ml resin), and the column is washed with 120 ml of the same buffer. The protein, which is adsorbed by the resin, is devoid of follicle-stimulating activity. The effluent obtained with the pH 5.1 buffer is brought to 3 M $(NH_4)_2SO_4$ by addition of solid $(NH_4)_2SO_4$. The precipitate formed is dissolved, dialyzed and lyophilized. The lyophilized product (*Fraction CU*) weighs 0.14 g and has an activity of 346 slope units/mg. *$(NH_4)_2SO_4$ Fractionation.* 0.14 g of Fraction CU is dissolved in 15 ml of distilled water and fractionated with $(NH_4)_2SO_4$ according to the method described above for preparation of Fraction SII. The yield of the active fraction is 0.1 g; it possesses an activity of 464 slope units.

Table I summarizes the purification procedure together with the yield and potency of each fraction. A 15-fold increase in biological activity can be achieved by the procedure with a yield of 100 mg of purified FSH from 10 g of lyophilized pituitaries; the follicle-stimulating activity of the product (*Fraction F*) has been assayed in graded doses by the augmentation test(5) and results are given in Table II. It is evident that the preparation contains a potency of approximately 600 slope units/ml, an activity comparable to that of ovine FSH preparation obtained by zone electrophoresis on starch(9).

When Fraction F was assayed in hypophysectomized male rats(10), the preparation elicited an increase of ventral prostate weight from 7 mg to 15 mg at a total dose of 0.1 mg, indicating that the fraction is contaminated with ICSH.¶ The preparation is practically free from thyrotropic, lactogenic, adrenocorticotropic and growth-promoting activities, according to tests by conventional assay procedures. Physicochemical investigations indicate that Fraction F is not homogeneous; further purification of this fraction is in progress.

TABLE II. Follicle-Stimulating Activity of Purified Human FSH (Fraction F) According to Augmentation Test.

| Total dose of fraction F, μg* | No. of rats | Body wt, g | | Ovarian wt, mg | |
|---|---|---|---|---|---|
| 0 | 11 | 52.4 | 1.0† | 30.5 | 2.3 |
| 25 | 9 | 49.1 | .8 | 48.9 | 3.0 |
| 50 | 13 | 54.2 | 2.6 | 61.0 | 4.1 |
| 100 | 11 | 51.8 | 1.3 | 79.5 | 3.5 |

* A constant daily dose (20 μg) of HCG was also administered to all experimental animals.
† Mean ± stand. error.

§ Composition of the buffer is as follows: 0.052 M $NaH_2PO_4$, 0.002 5 M $Na_2HPO_4$ and 0.45 M $(NH_4)_2SO_4$.

‖ For conditions used and for details of the operation of the resin colum, see Papkoff and Li(8).

¶ Purification of ICSH from human pituitary glands will be reported elsewhere; a total dose of 0.005 mg of purified human ICSH will elicit an increase of 100% in weight of ventral prostates of hypophysectomized rats over the controls.

*Summary.* A procedure for preparation of follicle-stimulating hormone from human pituitaries has been described. Approximately a 15-fold purification could be achieved by a procedure involving fractionation with $(NH_4)_2SO_4$ and a cation exchange resin.

*Addendum:* FSH fraction SII has been tested for gonad-stimulating activities in human subjects by Dr. D. M. Bergenstal of National Cancer Institute, Bethesda, Md. A female subject (age 32), one day after cessation of menstruation, was injected subcutaneously with 2.5 mg FSH concentrate every 6 hours for 6 days and followed by castration. Ovaries were highly stimulated with the appearance of multiple follicular cysts. Details of these clinical studies will be reported by Dr. Bergenstal elsewhere.

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## Association of Pyridoxine Deficiency and Convulsions in Alcoholics.*† (24203)

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Alcoholic withdrawal syndrome includes convulsions. It is generally believed that these seizures relate to idiopathic or post-traumatic epilepsy or alcohol withdrawal(1,2). As infants fed a formula deficient in Vit. $B_6$ (3) or adults with chemically induced Vit. $B_6$ lack (*i.e.*, administration of isoniazide(4) or desoxy-pyridoxine(5,6)) have occasional *grand mal* seizures, we wondered if seizures in alcoholics might not be associated with Vit. $B_6$ deficiency, particularly as their diets are grossly inadequate. The term "rum fit" has been used to describe these seizures occurring in the immediate 36- to 72-hour period after cessation of an alcoholic spree. Henceforth this nomenclature will be used to describe these patients. This study purports to determine if rum fitters may be Vit. $B_6$ deficient.

*Materials and methods.* Five rum fitters, 2 patients with alcoholism and associated epilepsy§, 6 patients in various phases of alcoholism but without fits, and 1 normal non-alcoholic individual were studied. The group consisted of 13 males and 1 female. All were seen immediately on admission to the hospital. They were given a Vit. $B_6$-free diet consisting of "Dexin"‖ in water mixture and daily vitamin supplement excluding Vit. $B_6$.¶ An initial urine was collected. Following this, the patients were fed 10 g dl-tryptophane mixed with Dexin in water, either orally or by gas-

* The authors wish to thank the house officers of the Harvard Medical Services at the Boston City Hospital for their cooperation in this study.

† This work was supported by grant from Nutrition Fn., N. Y., the Office of Surgeon General, Dept. of Army, and Merck Sharp & Dohme, Rahway, N. J., to Harvard University.

‡ Present address: Barnes Hospital, St. Louis, Mo.

§ These patients also had convulsions when not drinking and when their diets were good.

‖ A partial starch hydrolysate, Burroughs-Wellcome Co., Tuckahoe, N. Y.

¶ The daily vitamin supplement consisted of: thiamine 50 mg (i.m.) and 10 mg riboflavin, 100 mg niacin, and 100 mg ascorbic acid (p.o.).

TABLE I. Xanthurenic Acid Excretion before and after Pyridoxine.

| Diagnosis | Patient | Xanthurenic acid (mg/24 hr) after 10 g dl-tryptophane: No $B_6$ | After 100 mg pyridoxine HCl |
|---|---|---|---|
| Phases of alcoholism | | | |
| Acute and chronic ethanolism | 1* | 0 | 0 |
| Acute hallucinosis-tremulousness | 2* | 24.0 | 0 |
| Acute peripheral neuropathy | 3 | 0 | 0 |
| Wernicke-Korsakoff syndrome | 4* | 0 | 0 |
| | 5 | 55.0 | 0 |
| | 6* | 51.0 | 0 |
| Alcoholism with associated epilepsy | 7† | 0 | 0 |
| | 8 | 44.0 | 0 |
| Rum fitters | 9* | 110.0 | 0 |
| | 10† | 146.0 | 16.5 |
| | 11† | 158.0 | 20.0 |
| | 12† | 38.0 | 0 |
| | 13 | 0 | 0 |
| Normal non-alcoholic healthy | 14 | 0 | 0 |

* These patients in addition had the classical stigmata of cirrhosis.

† These patients exhibited cirrhosis + delirium tremens.

tric tube, and started on a complete 24-hour urine collection. Then 100 mg pyridoxine hydrochloride was injected intramuscularly and the study repeated for a second 24 hours. The volumes of these two 24-hour urine collections were measured and an aliquot saved, either refrigerated (20°C) or frozen (−4°C) until used. Electroencephalograms (EEG) were obtained in 5 of the 7 patients with *grand mal* seizures. Patients were allowed physiologic saline, hypotonic glucose, dilantin, thorzine, phenobarbital, or antibiotics as needed. No patient had known active tuberculosis. None was given isoniazide or iproniazide(4). Xanthurenic acid (XA) excretion for each of the 2 tryptophane load tests was measured(7). This method depends upon the reaction of XA with ferric ammonium sulfate in a "tris" buffer. The green color produced is proportional to the XA and is measured in the Coleman Junior spectrophotometer at 610 $\mu$. The pre-tryptophane urine sample from each patient was used as an internal colorimetric control.

*Results.* The Table records urinary XA of each patient and shows that the highest excretions occurred in rum fitters; lower values appear in the other 2 categories: alcoholism with associated epilepsy, and phases of alcoholism. After 100 mg pyridoxine intramuscularly, there was a marked decrease in XA excretions. The data are averaged and represented graphically. By comparison of average values for the 3 groups, the graph shows 4 times the XA excretion in rum fitters in the first tryptophane load test. The other 2 groups are similar.

XA excretion after a tryptophane load is accepted as the standard method for demonstrating Vit. $B_6$ deficiency in man(8). Rum fitters show the highest correlation with this evidence of Vit. $B_6$ lack. XA, where demonstrated, was in every case diminished by Vit. $B_6$. The small urine XA in non-rum fitters may well represent lesser degrees of Vit. $B_6$ shortage in sub-convulsive levels, as the dietary intake of these patients was also poor.

All values were calculated by the same standard curve at the same time. The oldest urine was 5 months old when its recorded value was obtained. XA varied somewhat with the standard curve, each set of reagents prepared, and to decrease in absolute value with storage. For example, levels of 600 mg and 225 mg XA in patients 10 and 11, respectively, were obtained when fresh urine was available. This would account for values being somewhat lower than those previously

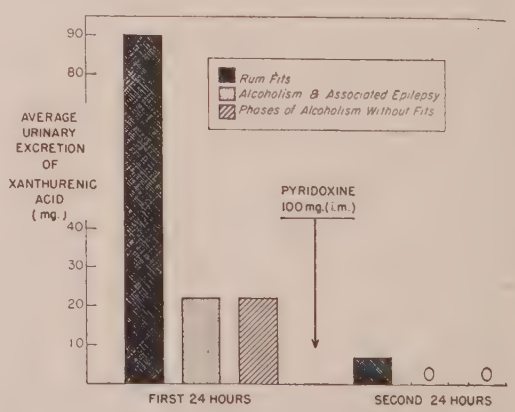


FIG. 1. Urinary excretion of xanthurenic acid after 10 g dl-tryptophane given at beginning of each 24-hr period. The first period is before and the second after administration of 100 $\mu$g pyridoxine.

reported in experimental Vit. $B_6$ deprivation in man(9).

The failure to demonstrate increased XA in urine after tryptophane loading does not exclude lack of pyridoxine group of vitamins. Another metabolite of tryptophane metabolism, such as 3-hydroxykynurenine, acetyl kynurenine or kynurenine, may have been the accumulated products in these cases(10). It is possible, as suggested by Roberts and Baxter(11), that the ultimate compound deficient in Vit. $B_6$ shortage is gamma amino butyric acid (GABA) which is formed by decarboxylation of glutamic acid, a reaction activated by pyridoxal-5-phosphate as coenzyme.

Three of the 5 rum fitters subsequently recovered through a stormy course with associated delirium tremens. One rum fitter presented as *status epilepticus*. The others had 1-2 isolated seizures. EEG's were normal in patients with rum fits. One of the 2 patients with alcoholism and associated epilepsy had an abnormal EEG (No. 7). All rum fitters showed multiple signs suggestive of deficiency of members of the Vit. B complex group of vitamins, the most common was depapillation and reddening of tongue, diffusely reddened oral mucous membranes, and injected conjunctivae. None showed evidence of scurvy.

Convulsions in alcoholics have been related to alcohol withdrawal and to idiopathic or post-traumatic epilepsy. It has been possible to separate a group of these patients who exhibited Vit. $B_6$ deficiency and who had no evidence of previous epilepsy, or of abnormal EEG's.

*Summary.* 1. Increased xanthurenic acid excretion after 10 g dl-tryptophane was demonstrated in 3 of 5 patients with rum fits. This metabolic defect was corrected by Vit. $B_6$ in a second tryptophane load test. 2. Patients with alcoholism and associated epilepsy, acute and chronic alcoholism, cirrhosis, acute hallucinosis-tremulousness, acute peripheral neuropathy, Wernicke-Korsakoff syndrome, and a non-alcoholic, healthy individual did not demonstrate significant Vit. $B_6$ deficiency by this test. 3. It is postulated that Vit. $B_6$ deficiency is etiologically related to rum fits.

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## Experimental Lathyrism in the Rat. Nature of Defect in Epiphysial Cartilage. (24204)

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Profound alterations have been described in the cartilages, bones and aortas of animals fed the seeds of *lathyrus odoratus* or treated with certain chemical compounds such as beta-aminopropinonitrile (BAPN), aminoacetonitrile (AAN)(1), and most recently semicarbazide(2). No agreement has been reached concerning the basic metabolic defect

TABLE I. Hydroxyproline (Collagen) Content of Epiphysial Cartilage from AAN-Treated and Control Animals.

| AAN-treated | | | | Control | | | |
|---|---|---|---|---|---|---|---|
| Wt dry cartilage (mg) | Hydroxy-proline content ($\mu$g) | Collagen content ($\mu$g) | % collagen | Wt dry cartilage (mg) | Hydroxy-proline content ($\mu$g) | Collagen content ($\mu$g) | % collagen |
| 1.3 | 3.2 | 240 | 18.6 | 3.0 | 6.6 | 494 | 16.5 |
| 1.45 | 3.8 | 282 | 19.5 | 1.5 | 3.4 | 257 | 17.1 |
| 1.2 | 2.5 | 186 | 15.5 | 1.25 | 2.8 | 218 | 17.4 |
| 2.0 | 4.4 | 328 | 16.4 | 1.3 | 2.6 | 194 | 15.0 |
| 3.65 | 8.0 | 597 | 16.6 | 1.65 | 3.6 | 267 | 16.3 |
| 2.4 | 5.9 | 440 | 18.3 | .85 | 2.3 | 171 | 20.1 |
| | | Avg | 17.5 | | | Avg | 17.1 |

responsible for the lesions. Both mucopolysaccharide and collagen components of the tissues in question have been implicated(1). Several years ago one of us(3) called attention to an extraordinary physical transformation of the epiphysial cartilage of animals fed sweet pea seeds, stating that "the epiphysial cartilage has become a sort of pulpy mass which appears in the fresh state, under the microscope, so lacking in cohesion that individual cells are found floating about by themselves." Subsequent observations of cartilage from animals which had received BAPN, AAN, and semicarbazide have confirmed this observation. The conclusion has seemed inescapable that, in comparison with fresh normal epiphysial cartilage which has a certain physical stability largely due to presence of collagen fibrils in the matrix, cartilage from treated rats might be deficient in this important fibrous protein. The experiments described herein, which were undertaken to demonstrate such a defect, have led us to postulate an entirely different concept concerning the change in epiphysial cartilage injured by lathrogenic materials.

*Materials and methods.* Young albino rats, approximately 40 g in weight, were given 20 mg AAN* by daily gavage. Although alteration in epiphysial cartilage can be detected as early as 24 hours, the optimal interval for maximum change is about 48 hours. The epiphysial cartilage, which by this time has become a mushy mass, was dissected away from the bone and perichondrial tissues under a binocular microscope, dried over phosphorus pentoxide and weighed on a torsion balance. For chemical determinations, samples were hydrolyzed in 6N HCl at 138°C for 3 hours. A modified(5) Neuman and Logan(4) procedure for hydroxyproline was then carried out. Collagen is expressed in terms of hydroxyproline by multiplying the amount of the latter by the factor, 7.46(4). For the centrifugation and electron microscopic studies known amounts of tissue were placed with 2 cc of distilled water in a plastic tube and disintegrated at 1°C with a microhomogenizer (Lourdes). The homogenate was then placed in tubes of the SW-39 rotor of the preparative model "L" Spinco centrifuge and spun at 5,000 rpm for 10 minutes, 10,000 rpm for 30 minutes, and finally 30,000 rpm for 30 minutes. The sediments of each centrifugation were resuspended in distilled water; drops of these and the final supernate were placed on grids coated with formvar or parlodion films, dried and shadowed with uranium at angles of arc tan 1/10. Electron microscopic observations and micrography were carried out with an RCA-electron microscope EMU-3B. Epiphysial cartilage from normal control animals was treated in similar fashion for chemical and electron microscopical studies.

*Results.* Chemical analysis of cartilage, freed of bone and perichondrial elements, from treated and control animals is shown in Table I. The normal collagen content, based on hydroxyproline determination of this tissue, is about 16-18% on a dry weight basis. It will be noted that little difference from this is found in animals receiving the lathyrogenic compound. This might lead one to assume

* Kindly supplied by Dr. I. V. Ponseti.

that the collagen content of the cartilage from AAN-treated rats in unaffected. Such an assumption is contrary to the gross appearance of the cartilage which has been described

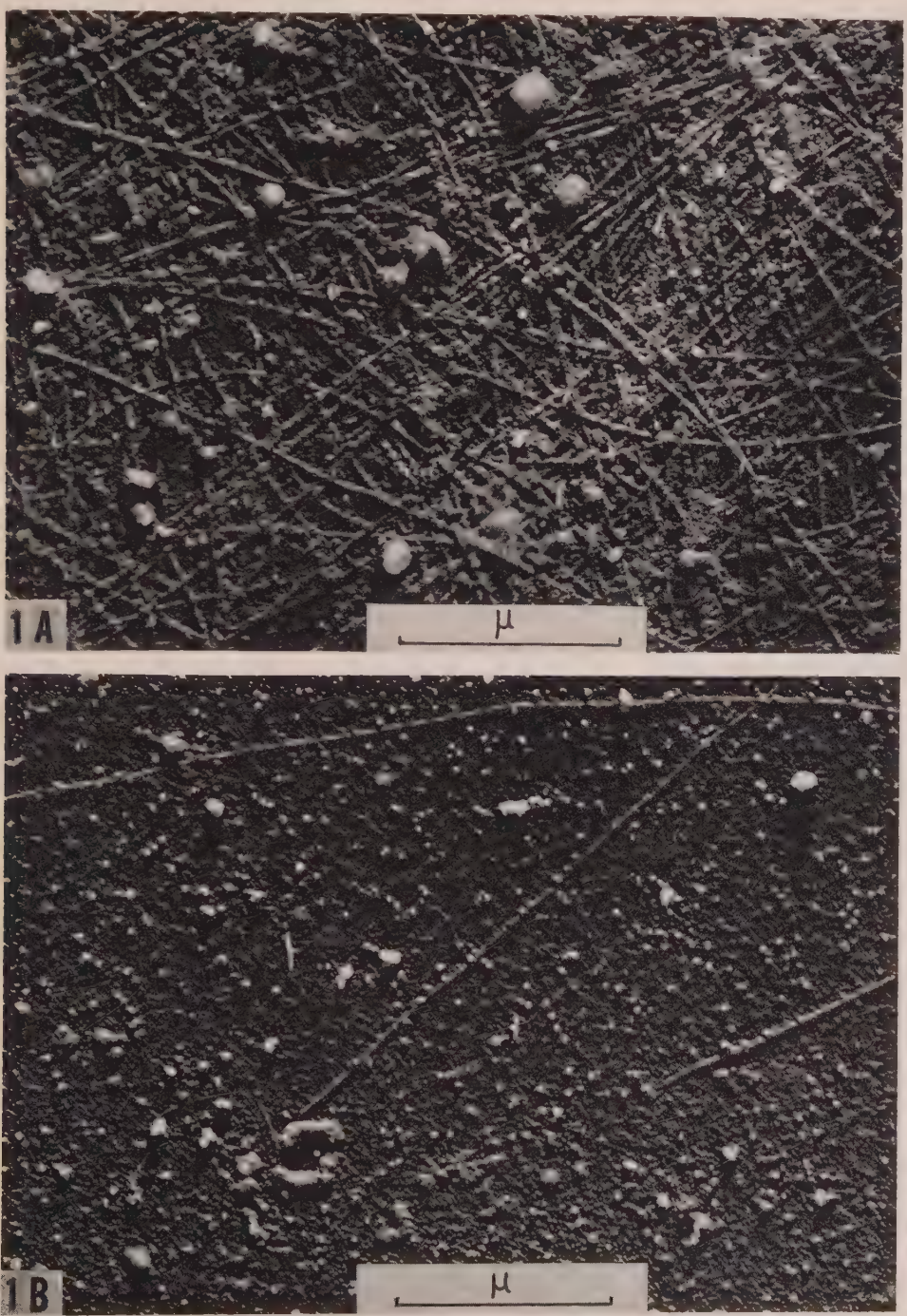


FIG. 1. Electron micrographs of rat epiphysial cartilage matrix. Sediment of 30,000 rpm suspended in water, air-dried on parlodion film, shadowed with uranium ($\tan^{-1}$ 1/10). (A) normal control and (B) AAN-treated animal, ×31,200.

above. The following studies of centrifuged fractions of homogenized cartilage are pertinent.

Electron microscopical observations of the sediments of 5, 10, and 30 thousand rpm from normal animals revealed fibrils ranging in width from 180A to 220A with varying lengths. Many of these fibrils were more than 80 $\mu$ long. Some of the fibrils showed periodicities of about 100-135A and 180-200A, while others did not suggest any periodicity in these airdried unstained shadowed specimens.

Fig. 1A shows the dense network of filaments found in the sediment of 30,000 rpm from the normal controls. In its supernate, Fig. 2A, fibrils are virtually absent, though a high concentration of particles is seen. The majority of these particles have widths of 100-125A or less; others vary from 250A to as high as 1500A. The appearance of the larger particles suggests that they are aggregates of the small ones since the majority of this size would have been sedimented at this centrifugal field, assuming a 1.2 g/cc particle density.

In contrast to these observations the number of fibrils in the sedimentation fractions from the cartilage of the AAN-treated animals is considerably reduced. Fig. 1B shows filaments from the sediment of the 30,000 rpm fraction of AAN treated animals with widths in the range of 100A to 200A and particles with widths of about 120A or more. In contrast to the normal controls the 30,000 rpm supernate of the treated rats in the majority of experiments appeared to contain a larger number of particles of about 40-90A in width and others in the range of 200-500A as seen in Fig. 2B. Many of the smaller particles appear to aggregate uniformly as seen throughout this electron micrograph.

*Discussion.* Examination of epiphysial cartilage in the fresh state clearly shows the physical deterioration which can be induced in a few days by feeding sweet pea seeds or by administration of lathyrogenic agents. This alteration appears to be associated with normal growth of cartilage, which we (unpublished observations) and others(1) have noted. Since the growth cycle of the cells of the epiphysial cartilage of rats at this age is about 48 to 60 hours(6), it is clear that 2 days of treatment would be sufficient for formation of new, diseased matrix which then might be expected to exhibit the alterations described above. The few fibers which are present doubtless date from the period before treatment was begun. It is also possible that some new fibers are formed, though not to the extent seen in the normals.

The pertinent literature on the pathogenesis of the changes in cartilage and other tissues has been reviewed(1). Several recent contributions are of interest in light of the observations presented herein. When subcutaneous abscesses are produced in rats, there is a delay in appearance of mature collagen and an abnormal persistence of amorphous intercellular material in AAN-treated animals as compared with controls(7). When incisions are made in the skin of rats, there is decreased formation of collagenous fibers in animals treated with lathyrogenic agents(8). Finally, in bone and cartilage from chicks treated with BAPN no alterations in hydroxyproline, hence collagen, content have been detected, though an increase in solubility of components of cartilage in 1M sodium chloride has been noted (Levene, C. I., Fed. Proc., 1958 v17, 445, abstract). All of these observations are in keeping with the findings we have described in epiphysial cartilage.

In order to have collagen fiber formation several conditions must be fulfilled. First, the constituent amino acids must be in optimal concentrations; second, they must be in proper alignment and linkage to produce the basic collagen molecule (tropocollagen)(9). These molecules then aggregate to form mature collagen fibers. Fiber formation *in vitro* has been described to result from the action of certain physiochemical procedures on the tropocollagen molecule(9). The *in vivo* mechanism of fiber formation is not as yet understood. The observations presented herein would appear to implicate the mechanism responsible for transformation of tropocollagen into mature collagen molecules. The basic defect, whether physical or enzymatic,

remains to be elucidated. The possible role of mucopolysaccharide in this phenomenon must be kept in mind(10).

*Summary.* The hydroxyproline, and hence

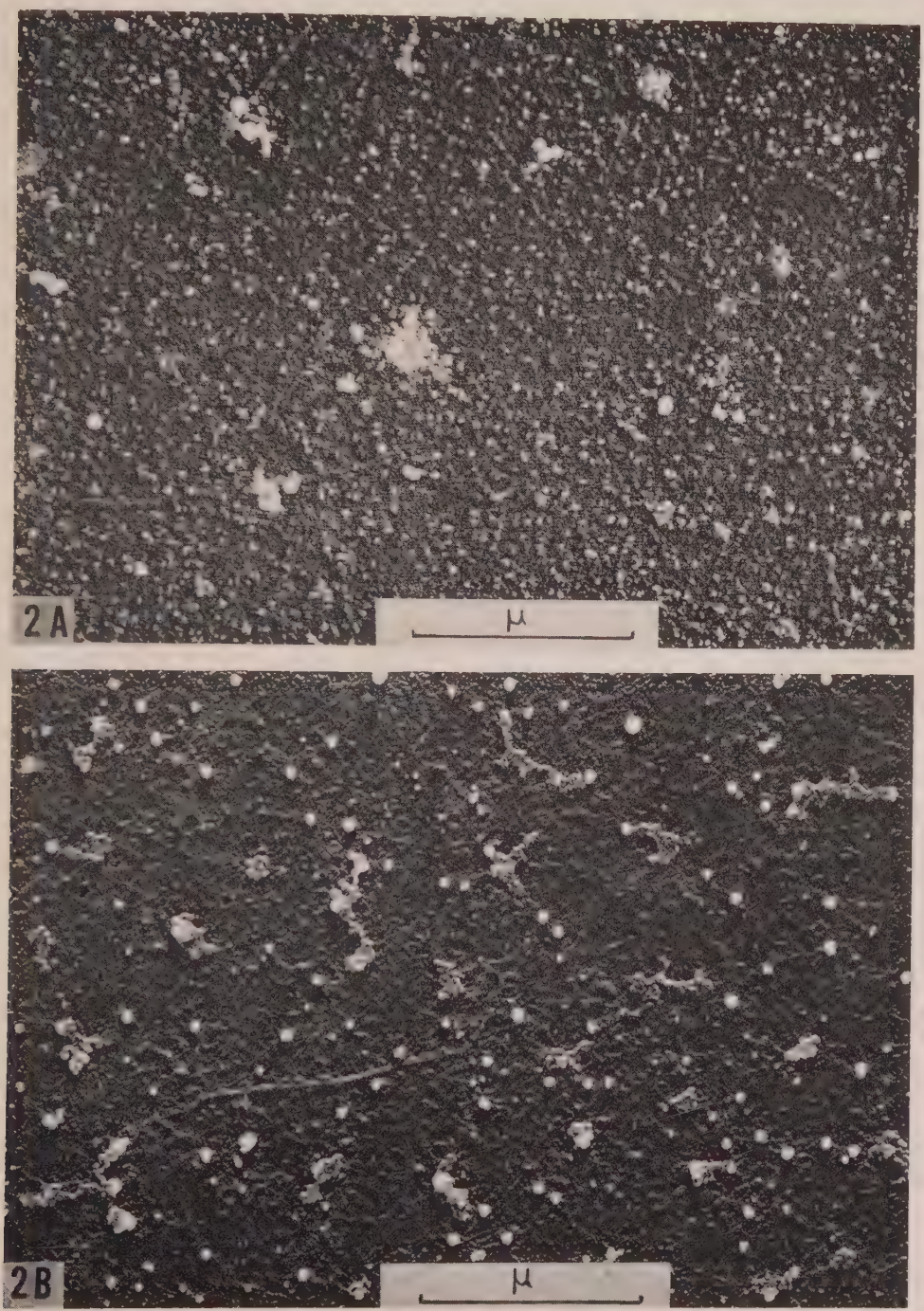


FIG. 2. Electron micrographs of rat epiphysial cartilage matrix supernate of 30,000 rpm fraction No. 4 in distilled water. Air-dried on parlodion film, shadowed with uranium ($\tan^{-1}$ 1/10). (A) from normal control and (B) AAN-treated rat, ×31,200.

collagen, content of epiphysial cartilage from rats treated with AAN is not reduced from the normal. Homogenized fractions of cartilage from rats which have received AAN are virtually devoid of collagen fibrils when examined with electron microscope. The defect appears to be a failure of the tropocollagen molecule to form collagen fibers.

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## Cytopathogenicity in Tissue Cultures by a Tumor Virus from Mice. (24205)

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A virus, which we shall refer to as SE polyoma virus, was recovered from tissue cultures inoculated with tumor material from mice and was shown to induce multiple tumors in mice(1,2,3) and hamsters(4). The present report describes cytopathic effects which appear to be produced by the SE polyoma virus in tissue cultures. The results so far obtained show that these effects parallel the capacity of tissue culture fluids to induce tumors in hamsters.

*Methods.* Tissue cultures from embryos of Swiss mice were propagated in 2 ounce prescription bottles by modification of the trypsin digest method of Youngner(5). Synthetic medium 199(6) containing 2% inactivated calf serum was used to initiate growth, and medium 199 with 1% inactivated rabbit serum was used as maintenance medium. For quantitative titration or serum-virus neutralization tests the cells were retrypsinized with 0.25% trypsin in Earle's balanced salt solution(7) at 35°C for 15 to 20 minutes, centrifuged, washed, resuspended in initial medium and dispensed in roller tubes. The cells in the retrypsinized cultures were clear and uniform. The strain of the agent used, originated from liver and spleen of a single AKR mouse with spontaneous leukemia and was passed serially into 3 newborn mice, with intervening passages in tissue cultures, followed by 2 to 4 passages in newborn hamsters with intervening tissue culture passages. Each passage animal had developed neoplasms. Tumor tissue from only one animal from each litter was used for passage. Titration of virus at several passage numbers was made in roller tube cultures of retrypsinized mouse embryo cells by preparing decimal dilutions and inoculating 4 tubes/dilution with 0.5 ml each. The cultures were refed at weekly intervals with 2 ml of nutrient fluid. Serum-virus neutralization tests were also carried out in roller tube cultures. Mixtures of equal volumes of 10-fold dilutions of the polyoma virus and 1% antipolyoma virus rabbit serum and mixtures of similar dilutions of virus and normal rabbit serum, were held at 36°C for 4 hours before being added to the roller tubes. The antiserum was made by hyperimmunizing rabbits with fluids from tissue cultures infected with the virus. The tubes were refed after one week with medium containing 1% immune and normal rabbit serum respectively. The final microscopic examinations were made at 3 weeks. To demonstrate induction of tumors, hamsters 1 to 3 days of age were injected with 0.2 ml doses of tissue culture fluid

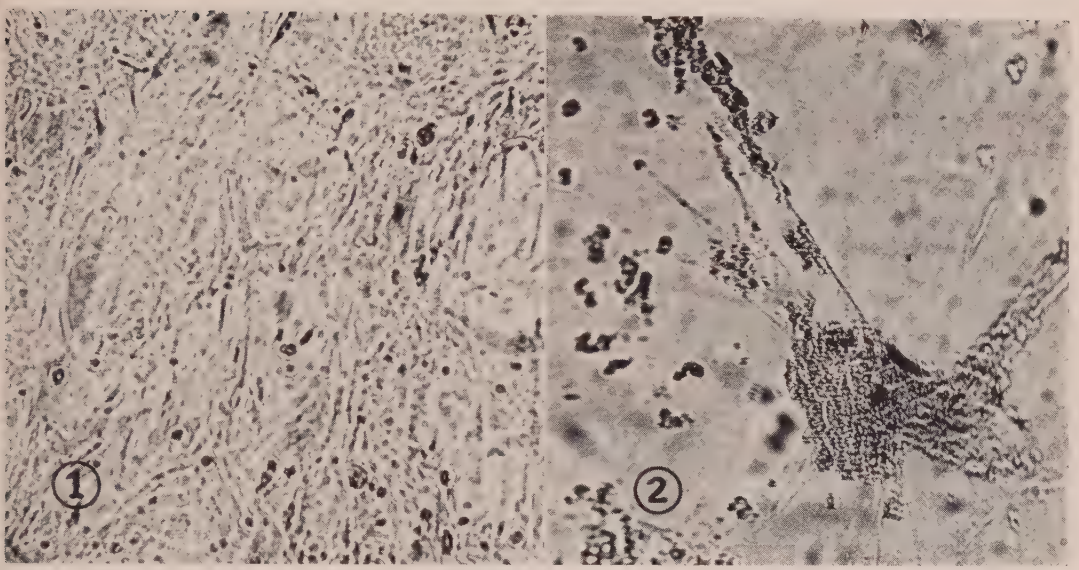

FIG. 1. 19-day-old culture of mouse embryo cells. Unstained. × 60.
FIG. 2. Cytopathic changes in mouse embryo cells 14 days after inoculation with SE polyoma virus. Unstained. × 60.

subcutaneously. The young were divided among mothers as equally as possible so that each set of hamsters inoculated were the same age.

*Results.* During many early tissue culture passages of the virus, infected cultures did not appear to be different from the uninfected control cultures; in others slight cytopathogenic changes occurred in infected cultures at irregular intervals in 2- to 3-week-old cultures. As tissue culture or animal passages were continued these cytopathogenic changes in tissue cultures occurred regularly and degeneration was more extensive.

The changes consisted of patches of small dark cells which appeared on sheets of normal appearing cells after 4 to 7 days. The number of these pyknotic cells continued to progress in 1 to 2 weeks until the majority of cells were effected, causing many of them to fall from the glass (Figs. 1 and 2). Subpassages of fluids from such cultures regularly produced cytopathic changes in other mouse embryo cultures.

Titrations showed a $TID^{50}$ of approximately $10^{-3}$ at 2 weeks and $10^{-4}$ or $10^{-6}$ at 3 weeks. The endpoints were more distinct at 3 weeks.

Reduction of the titer of the virus was noted when cultures were inoculated with mixtures of the virus and 1% homologous antiserum against the polyoma virus, as compared to similar cultures inoculated with mixtures of virus and normal rabbit serum. Degenerative changes were noted in cultures with undiluted and $10^{-1}$ virus combined with immune rabbit serum but not at higher dilutions. Titration of the virus with normal rabbit serum showed cytopathic changes through the $10^{-5}$ dilution. Immune rabbit serum was effective in preventing induction of tumors when combined with virus before administration to newborn mice(8).

Infectivity for hamsters appeared to be correlated with cytopathogenic changes in tissue cultures. To demonstrate this, infected fluids from tissue cultures, which showed cytopathogenic changes as a result of being incubated with a $10^{-5}$ dilution of virus and fluid from cultures incubated with a $10^{-6}$ dilution of virus which did not cause degenerative changes, were injected into hamsters. The results of a single experiment are given in Table I. All hamsters inoculated with fluid from cultures that failed to show degenerative changes were free of tumors until the 34th day, and 6 of the 11 were free of tumors on the 46th day. Two hamsters were missing on the 34th day.

TABLE I. Effect of Inoculating Hamsters with Supernatant Fluid from Tissue Cultures Which Showed Cytopathic Changes, and with Fluid from Cultures Which Did Not Show Such Changes.

| Virus in tissue cultures | | | No. of hamsters | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | At 26 days | | | At 43 days | | |
| Inoculum | Cytopathogenicity | Inoculated | Lost | With tumors | Surviving and free of tumors | Lost | With tumors | Surviving and free of tumors |
| $10^{-6}$ | + | 12 | 7 | 5 | 0 | 0 | 0 | 0 |
| $10^{-6}$ | 0 | 11 | 0 | 0 | 11 | 2 | 3 | 6 |

TABLE II. Results of Infecting Hamsters with SE Polyoma Virus Filtered through Gradocol Membranes and with Supernatant Fluids of Tissue Cultures Incubated with the Filtrates.

| Pore size of filter, mμ | No. of passages of filtrates in tissue culture | Cytopathogenicity | No. of hamsters | | | |
|---|---|---|---|---|---|---|
| | | | Inj. | Cannibalized | With tumors | Surviving and free of tumors |
| Unfiltered | none | | 8 | 3 | 3 | 2 |
| 220 | " | | 8 | 0 | 1 | 7 |
| 120 | " | | 10 | 0 | 3 | 7 |
| " | 2 | + | 9 | 1 | 8 | 0 |
| " | 3 | + | 13 | 9 | 4 | 0 |
| 77 | none | | 9 | 0 | 0 | 9 |
| " | 4 | 0 | 12 | 0 | 0 | 12 |
| 43 | none | | 11 | 0 | 0 | 11 |
| " | 4 | 0 | 26 | 0 | 0 | 26 |

On the other hand all 12 animals inoculated with fluid from cultures which showed cytopathic changes, had tumors or were dead 26 days after injection. Seven were cannibalized on 17th to 21st day and were not examined.

Evidence of correlation of cytopathogenicity and tumor induction was also obtained from ultrafiltration experiments designed to establish size of the infective agent.

Virus-infected fluids were filtered through a Seitz S3 filter and a gradocol membrane of 590 mμ porosity. Portions of this filtrate were then filtered through 4 membranes of different pore size (Table II). Fluid passed through membranes of 220 and 120 mμ average pore size caused cytopathic changes in tissue cultures and hamsters inoculated with these filtrates or with filtrates after passage in tissue cultures developed tumors.

The fluid passed through membranes of 77 and 43 mμ porosity did not cause cellular changes in tissue cultures. These filtrates or the fluid from tissue cultures inoculated with these filtrates, failed to induce tumors in hamsters after 128 days.

The results thus far are entirely compatible with the hypothesis that the tumor virus causes cytopathic changes in mouse embryo tissue culture. Because of the possibility of the presence of a concomitant virus in any tissue culture system, further experiments on correlation of tumor induction and cytopathic changes in tissue cultures are in progress. The practical importance of a tool to determine capacity of a fluid to induce tumors is obvious and prompts this early report.

*Summary.* Cytopathogenic changes were observed in mouse embryo cell cultures that had been inoculated with tissue culture-grown virus isolated from mouse tumor tissues. Quantitative assays of the virus, referred to as the SE polyoma virus, and serum-virus neutralization tests were correlated with the induction of tumors in hamsters.

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## Effect of Major Dietary Constituents on Growth Response of Rats to Vit $B_{12}$.* (24206)

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Hartman *et al.*(1) reported that high protein rations produced a deleterious and sometimes fatal effect in Vit. $B_{12}$ deficient rats. Bosshardt *et al.*(2) found that rations high in fat had a sparing action in mice upon the requirement for Vit. $B_{12}$. McCollum and Chow(3) reported that casein rations high in carbohydrate increased the Vit. $B_{12}$ deficiency in female rats. The purpose of this study was to determine which of the 3 basic dietary constituents—fats, carbohydrates, and proteins—would produce the greatest growth response to Vit. $B_{12}$ when plant protein rations were used.

*Method.* Adult female Sprague-Dawley rats (100 days old) were placed on a corn-soybean ration(4) at mating and maintained on this ration during pregnancy and lactation. The young had access to this ration until they were placed on experimental rations at weights of 40 ± 5 g. The care and handling of animals have been described previously(4). The rations (Table I) were adjusted so that the same percentage of calories was derived from protein in the high carbohydrate and high fat rations. Protein was isocaloric in the high protein and high protein-high fat rations. All animals received, in addition, 2 drops of Halver Oil weekly.

*Results.* The results are tabulated in Tables II and III. The differences in growth between the supplemented and unsupplemented rats on the high carbohydrate and high fat rations were not great. Similar results were also obtained in rats on the low protein diet in Series I. Growth differences obtained between supplemented and deficient animals fed the high protein and high protein-high fat diets were in every instance highly significant ($P<0.01$). Large variations in growth occurred with these diets with the greatest variation in the non-supplemented groups.

TABLE I. Composition of Rations.

| Ingredients | High carbohydrate | High fat | High protein, high fat | High protein | Low protein |
|---|---|---|---|---|---|
| Drackett protein* | 24. | 35. | 66.5 | 54. | 16.2 |
| Corn starch | 62.5 | 16.5 | | 32.5 | 70.5 |
| Hydrogenated fat† | | 35. | 20. | | |
| Corn oil | 5. | 5. | 5. | 5. | 5. |
| Salts XIV‡ | 4. | 4. | 4. | 4. | 4. |
| Vit. mix§ | 2. | 2. | 2. | 2. | 2. |
| Alphacel‡ | 2. | 2. | 2. | 2. | 2. |
| Methionine | .5 | .5 | .5 | .5 | .5 |
| Vit. $B_{12}$ mix‖ when indicated | .1 | .1 | .1 | .1 | .1 |

* Drackett Assay Protein G-1 81% protein (N × 6.25). † Crisco. ‡ Nutritional Biochemicals. § *J. Biol. Chem.*, 1954, v206, 765. ‖ 5% 0.001 Triturate $B_{12}$ (Merck), 95% sucrose.

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TABLE II. Effect of Diet on Growth Response of Rats to Vit. $B_{12}$.

| Ration | Avg growth in g, 5 wk | | | | | | Diff. in g | |
|---|---|---|---|---|---|---|---|---|
| | $-B_{12}$ | | | $+B_{12}$ | | | | |
| **Series I ♂** | | | | | | | | |
| High Carbohydrate | 161 | 10.4* | (8)† | 183 | 4.0 | (7) | 22 | 10.5‡ |
| Fat | 177 | 8.8 | (8) | 212 | 4.1 | (7) | 35 | 9.7 |
| Protein | 117 | 12.4 | (8) | 180 | 5.2 | (7) | 63 | 13.4 |
| Protein, High Fat | 93 | 10.4 | (8) | 174 | 5.7 | (7) | 81 | 11.9 |
| Low Protein | 164 | 3.4 | (10) | 176 | 2.2 | (10) | 12 | 4.0 |
| **Series I ♀** | | | | | | | | |
| High Carbohydrate | 106 | 5.3 | (8) | 119 | 3.4 | (7) | 13 | 6.3 |
| Fat | 114 | 5.6 | (8) | 131 | 4.0 | (7) | 17 | 6.8 |
| Protein | 96 | 7.8 | (8) | 122 | 3.4 | (7) | 26 | 8.5 |
| Protein, High Fat | 89 | 13.7 | (8) | 132 | 4.8 | (7) | 43 | 14.5 |

* Stand. error of mean. † Figures in parentheses indicate No. of animals. ‡ Stand. error of diff.

TABLE III. Effect of Diet on Growth Response of Male Rats to Vit. $B_{12}$.

| Ration | Avg growth in g, 5 wk | | | | | | Diff. in g | |
|---|---|---|---|---|---|---|---|---|
| | $-B_{12}$ | | | $+B_{12}$ | | | | |
| **Series II** | | | | | | | | |
| High Carbohydrate | 132 | 2.9* | (6)† | 147 | 2.9 | (6) | 15 | 4.1‡ |
| Fat | 178 | 9.9 | (6) | 187 | 6.4 | (6) | 9 | 11.8 |
| Protein | 124 | 13.8 | (6) | 172 | 4.5 | (6) | 48 | 14.5 |
| Protein, High Fat | 149 | 9.4 | (6) | 185 | 7.4 | (6) | 36 | 12.0 |
| **Series III§** | | | | | | | | |
| High Carbohydrate | 100 | 3.0 | (6) | 113 | 4.8 | (6) | 13 | 5.6 |
| Fat | 106 | 4.0 | (6) | 115 | 4.8 | (6) | 9 | 6.2 |
| Protein | 56 | 5.5 | (6) | 89 | 5.9 | (6) | 33 | 8.1 |
| Protein, High Fat | 73 | 6.0 | (6) | 95 | 1.9 | (6) | 22 | 6.2 |

* Stand. error of mean. † Figures in parentheses indicate No. of animals. ‡ Stand. error of diff. § Three wk growth period in Series III.

*Discussion.* The greater growth differences observed in rats on high protein diets were in accord with the work of Hartman *et al.*(1). Numerous reports have implicated Vit. $B_{12}$ in protein metabolism(5,6). In recent studies a decreased incorporation of labeled amino acids into protein was observed in Vit. $B_{12}$ deficient animals(7). On a growth basis it appears that rats on adequate protein intake (high carbohydrate and high fat diets) utilized protein almost to the same extent with or without Vit. $B_{12}$. Since the lack of Vit. $B_{12}$ was more detrimental to growth of rats on high protein rations, it apears that this vitamin plays a role in the utilization of excess protein for non-protein functions.

The fat sparing action found in mice by Bosshardt *et al.*(2) and in rats by McCollum and Chow(3) was seen in Series II and III; however, high levels of fat appeared to increase the deficiency in rats fed the high protein rations in Series I. Spivey *et al.*(8) found that high fat diets increased the Vit. $B_{12}$ deficiency in chicks. McCollum and Chow(3) and Cuthbertson *et al.*(9) demonstrated that high carbohydrate diets produced a greater Vit. $B_{12}$ deficiency in rats than high protein or high fat diets. In our work, less growth difference was observed with rats on high carbohydrate rations. These contrasting results might be explained on the basis of different type rations used, and the relative degree of coprophagy practiced. It has been shown that the nature of the ration probably has an influence on the intestinal synthesis of Vit. $B_{12}$ and thus on the benefits of coprophagy(10). Barnes *et al.*(11) reported that the production of Vit. $B_{12}$ deficiency was closely related to the extent of coprophagy practiced by the rat.

*Summary.* 1) Rats born to dams maintained on Vit. $B_{12}$ deficient diets from mating

to weaning were fed rations high in carbohydrate, fat, protein, and protein and fat with and without Vit. $B_{12}$. 2) The greatest degree of deficiency was consistently observed in rats on high protein and high protein-high fat rations. Since Vit. $B_{12}$ increased ability of rats to utilize excess protein, one of the important functions of this vitamin appears to be the metabolism of excess amino acids for non-protein functions. 3) Differences in growth on various diets, however, may be due to degree of intestinal synthesis of Vit. $B_{12}$ and extent of coprophagy.

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## Cytopathogenic Effects of Hemadsorption Virus Type I.* (24207)

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Several viruses causing human disease induce formation of large multinucleated cells in tissue culture preparations. These include measles(1), mumps(2), croup associated virus(3-4), and 2 recently described myxoviruses. Chanock, *et al.*(5), have termed these latter hemadsorption viruses Types I and II. They were isolated from children with respiratory illnesses and appear distinct from previously described viruses. Attention was called to the fact that hemadsorption virus Type I has been isolated from uninoculated HeLa cell cultures. The unique cytopathogenic changes caused by this virus in HeLa cell cultures have been followed by time-lapse phase-contrast cinemicrography.

*Materials and methods.* Hemadsorption virus Type I (Mill's strain) was obtained from Dr. Wallace Rowe following its isolation from uninoculated HeLa cultures. The virus pool for these studies was second passage LoHi‡ virus titering approximately $10^5$ $T.C.D._{.50}$ per ml. Photographic chambers were constructed by attaching #1 cover slips on each side of perforated stainless steel slides. The top cover slip was placed slightly eccentrically, leaving a small opening at one edge for access to the chamber. This was subsequently sealed with a drop of paraffin. A suspension of small clumps of HeLa cells in Eagle's basal medium, 5% calf serum, 5% cord serum, penicillin 100 units and streptomycin 100 $\mu$g/ml, was introduced into the chamber which was then sealed and inverted until cell attachment occurred. These preparations were carried on growth medium until colonies reached optimal size for photography, at which time the growth medium was removed, the cells washed twice with buffered

* This work was supported in part by Grant from U. S. Public Health Service.

† Markle scholar in Medical Sciences.

‡ Designates a stable cell line established from explants of a lymph node from patient with rheumatoid arthritis. The strain grows on glass in an epitheloid pattern with a mean generation time of 24 hours. The viral spectrum is similar to that of HeLa. Tumors have been produced by inoculation of irradiated cortisone treated mice.

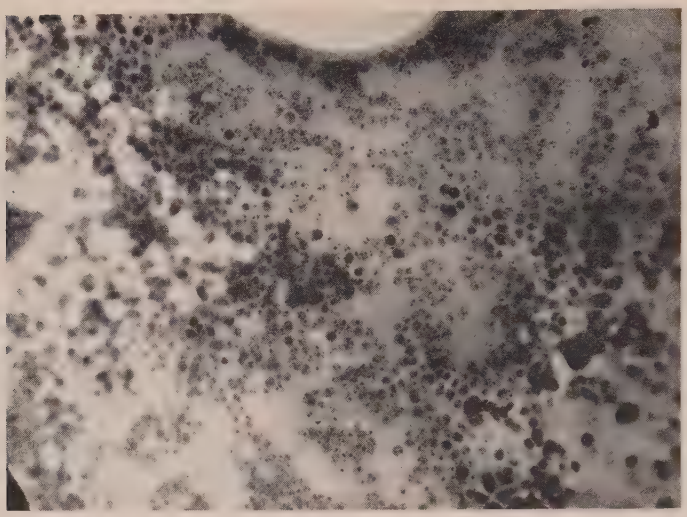

FIG. 1. Cytopathogenic effect of hemadsorption virus Type I 36 hr after infection. Hematoxylin and eosin. × 160.

salt solution, and the chamber inoculated with 0.1 ml of virus in 0.4 ml medium consisting of 78% Medium 199, 20% trytose phosphate, 2% calf serum, penicillin 100 units and streptomycin 100 μg/ml. Cytopathogenic changes were documented through an incubated phase-contrast microscope by intermittent photography at a speed of 4 frames/minute. Stained cultures were prepared by the colloidian membrane technic(1).

*Results.* Detectable changes occurred approximately 12 hours after inoculation although the time of initiation of changes in different preparations and in different colonies in the same chamber was variable. Early changes consisted of appearance of multinucleated cells which, through a process of coalescence of cytoplasm of adjacent cells, increased rapidly in size until hundreds of nuclei were included in a single cell (Fig. 1). An example of fusion of 2 cells is shown in Fig. 2. After a few hours the central area of many lesions detached from the glass and fell away into the medium leaving, characteristically, a hole lined by a syncytial-like layer of cells (Fig. 3). During 12 to approxi-

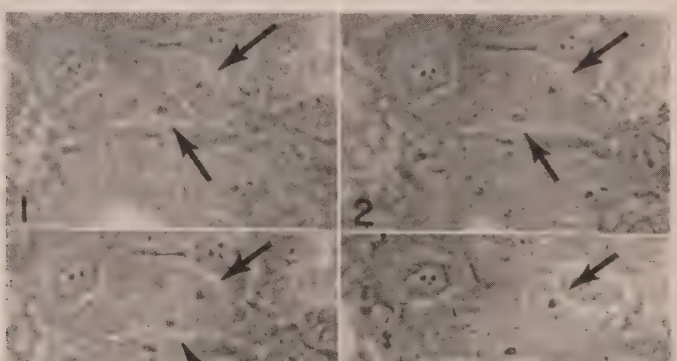


FIG. 2. Excerpts from motion picture record of HeLa cell fusion. Ten min. interval between frames. Frame 1—Arrows point to nuclei of 2 separate cells. Frame 2—Fusion has started at lower right border. Frame 3—Complete fusion. Frame 4—Nuclei have drifted apart. Living image. × 320.

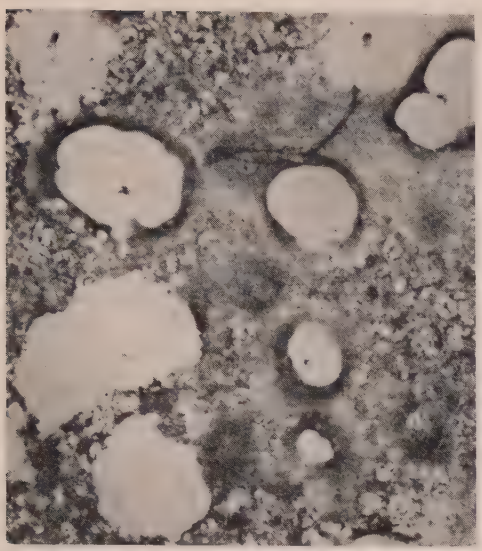

FIG. 3. HeLa culture 48 hr after infection with hemadsorption virus Type I. Hematoxylin and eosin. × 60.

mately 36 hours after infection when the marked cytoplasmic membrane changes were occurring, there was striking lack of morphological change in other organellae of the cells. The nuclei maintained their integrity and motility, including the rotary motion frequently seen in HeLa cells. Mitochondria similarly continued to exhibit essentially normal motility and morphology. Some decrease in nuclear size did occur late in the infection but by this time, distortion of the whole culture made further evaluation difficult or impossible.

We have not observed nuclear division in the absence of cytoplasmic division, although under low power several hundred nuclei in a single cell can be followed for many hours. It is quite possible that this process may occur, but our studies clearly rule it out as a major process in the development of the large multinucleated cells characteristic of infection with this virus. Mitoses within the developing multinucleated cells similarly have not been observed, but normal mitoses immediately adjacent to the developing lesion have been photographed and have been seen frequently in stained preparations.

Since the virus which we used had been isolated from presumably uninoculated HeLa cultures, and since both giant cells and multinucleated cells are seen in stock HeLa cultures, the possibility that these represented a limited viral effect was considered. However, on several occasions we have apparently mistaken such cells for early viral lesions and photographed them for hours with no evidence of progression, although progressive changes were occurring in other areas of the culture.

*Discussion.* Enders(6) noted that after inoculation of tissue culture preparations with measles virus, the appearance of multinucleated cells precedes by many days the production of inclusion bodies or other changes, and postulated that multinuclearity in this instance is due to coalescence of the cytoplasm of adjacent cells. Chanock(3) has described changes in croup associated virus inoculated cells quite similar to those described here. Henle *et al.*(2) have shown that cell fusion is the process responsible for multinuclearity in mumps infected cultures and postulates a relation between hemolytic and "cytolytic" properties of mumps virus. Hemadsorption virus Type I may cause hemolysis of red cells, but whether the remarkable effects on HeLa cell membranes is due to this property or to impairment of cellular mechanisms responsible for maintainance of membrane integrity is unknown.

*Summary.* Cytopathogenic effects of hemadsorption virus Type I in HeLa cells as studied by time-lapse phase-contrast cinemicrography and by colloidian membrane preparations have been described. The cytopathic changes are characterized by formation of large multinucleated cells followed by focal loss of cells leaving multiple holes in the cell sheet. Coalescence of the cytoplasm of adjacent cells precedes marked alteration of intracellular organellae by many hours.

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## Studies on Chloride of Bone in Cat and Rat.* (24208)

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The chloride space, calculated from values for bone chloride content, is frequently used as a measure of extracellular fluid volume of bone. Use of this calculation rests on the assumption that chloride is not located in cells or on crystals but solely in extracellular fluid. However, it has been observed by Nichols and Nichols(1) in dogs and by ourselves in rats, that the chloride space is often greater than one would expect if total bone water content was extracellular fluid. This discrepancy is complicated by the difficulties of chloride analysis in a tissue that contains small amounts of chloride with the possibility of associated errors due to blood content of cortical bone or contamination of bone samples with marrow and blood.

The purpose of this paper is to present the results, in cats and rats, of an accurate method of chloride analysis for cortical bone. Use was made of $Br^{82}$ to determine exchangeability of the chloride and as a check on the accuracy of chloride analysis. $Cr^{51}$ labeled red cells were also used to determine contributions made by blood content of bone to its water and chloride content.

*Procedure.* Cats of varying weights but unknown ages were anesthetized with Dial with Urethane (Ciba) injected intra-abdominally. Venesections were made in both saphenous veins. A solution of $KBr^{82}$‡ in normal saline and a suspension of $Cr^{51}$ labeled red cells were injected into one saphenous vein by the method of Barnett and Fellers(2), using a maximum of 8 ml of normal saline as wash solution. The other venesection was used solely for withdrawal of samples. Previously an aliquot of the $KBr^{82}$ solution had been removed for counting as a standard, leaving 10 $\mu$c to be injected. The $Cr^{51}$ labeled cell suspension was prepared by withdrawing 3 ml of blood and incubating it for 30 minutes at room temperature with approximately 100 $\mu$c of $Cr^{51}$ in the form of $Na_2Cr_2O_7$.§ Red cells were washed 3 times with normal saline, reconstituted to original volume, an aliquot removed for counting as a standard and the remaining cell suspension injected into the cat. Two hours were allowed for equilibration of both isotopes, since previously determined disappearance curves of both $KBr^{82}$ and $Cr^{51}$ labeled red cells, injected intravenously, reached a constant slope by 2 hours after injection. At the end of this period, blood was withdrawn for counting and analysis. At the same time the blood supply of left upper extremity was occluded with a tourniquet and the limb amputated at elbow. The radius and ulna were dissected free, the periosteum removed by scraping and the marrow blown out with compressed air. The bone was then broken into small pieces, cleaned of remaining marrow by blotting with filter paper, placed in a tared vial and weighed immediately. For determination of chloride content of rat bone, groups of Holtzman rats of known ages were exsanguinated after ether anesthesia. All long bones of young rats were removed but, in older animals, only the right upper and lower extremities were taken. Before weighing in tared bottles, the bones were cleaned using the same procedure as for cat bone.

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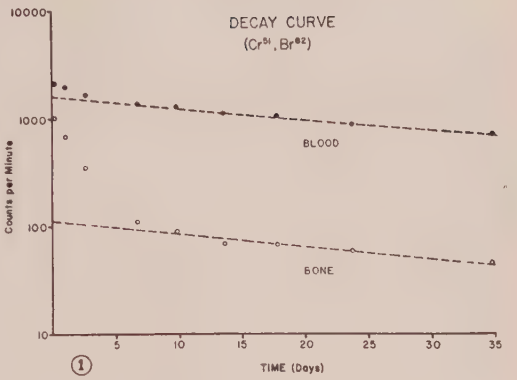
§ Rachromate, Abbott.

*Analytical methods.* Blood and plasma water were determined by drying. Plasma chloride was determined by Volhard titration as modified by Van Slyke(3). Bone water was determined by drying at 65°C *in vacuo* for 48 hours. Fat was extracted from cat bones with dry ethyl ether at room temperature but was not extracted from rat bone, since the fat content was less than 2% in preliminary extractions. The bones were then ground to a fine powder with a mortar and pestle, placed in platinum crucibles, redried and weighed. A modification of the method of Cheek and West(4) was used to determine bone chloride as follows: Approximately 0.5 ml of 2N KOH (Special pellets—Low in Chloride, Baker)/150 mg of dry bone was added to the crucible contents. The crucibles were placed on steam bath for at least one hour until dry and then in a muffle furnace at 550°C overnight. Upon cooling, $AgNO_3$-$HNO_3$ was added and the solutions titrated in duplicate with KCNS using Ferric Alum Indicator. These solutions were colorless and the end points distinct. A blank determination on the KOH revealed no detectable chloride. Ash content was determined by ashing another aliquot of bone powder at 525°C. Determination of radio-activity in the samples was made with a well-type, NaI crystal, scintillation counter. Counting geometry was maintained by using glass vials containing 2 ml of solutions or approximately 2 cc of dried bone chips. Corrections were made for resolving time of the counter but not for absorption in the samples. Initial counting of $Cr^{51}$ and $Br^{82}$ standards, blood, plasma and bone was done within 4 hours of obtaining the samples. Blood and bone samples were counted again at 24-hour intervals until the curve of decay indicated the only remaining radioactivity was from $Cr^{51}$. Four or 5 more counts were made at intervals of approximately 5 days.

*Calculations.* Extracellular fluid chloride concentration was calculated from serum chloride concentration using a Donnan factor of 0.977 and the observed serum water concentration in both cats and rats. The estimated interstitial chloride of bone was defined as the chloride that would be present if total bone water was an ultrafiltrate of plasma. This is



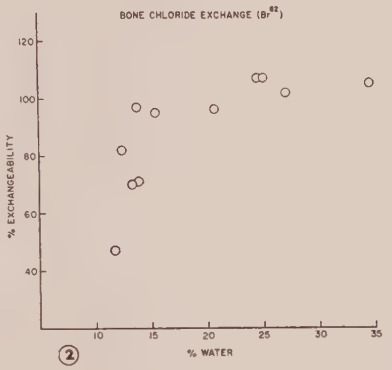




FIG. 1. Decay curve of $KBr^{82}$ and $Cr^{51}$ labeled red cells in aliquots of blood and bone from the same cat.

FIG. 2. Exchangeability of bone chloride determined with $Br^{82}$ plotted against water content of dry fat-free bone.

based on the assumption that intracellular water of bone is negligible and that the water of the hydration shells of the crystals is freely permeable to the ions of extracellular fluid. Estimated Interstitial Chloride = G $H_2O$/g dry bone x $(Cl)_E$ where $(Cl)_E$ is the extracellular chloride concentration. Excess chloride was then defined as the value obtained by subtraction of estimated chloride from observed chloride content. The content of organic substances was estimated as the difference between total bone weight and the sum of ash and water contents. Separation of $Br^{82}$ and $Cr^{51}$ in blood and bone could be made by virtue of the fact that these isotopes have widely differing half-lives ($Br^{82}$ 35.87 hrs, $Cr^{51}$ 27.8 days). Fig. 1 shows a typical decay curve for aliquots of blood and bone in one cat. By inspection a regression line passing through the last points is drawn back

through the ordinate. This line has a half-life of 28 days, that of $Cr^{51}$ and its intersection with the ordinate indicates counts/minute due to radioactivity of $Cr^{51}$ at the time of initial counting. The difference between this figure and the total counts/minute is the radioactivity from $Br^{82}$. Assuming the venous hematocrit to be equal to the hematocrit of the blood in cortical bone, the blood volume of bone was calculated from the equation,

$$\text{Blood volume (ml/g bone)} = \frac{Cr^{51}\text{/g bone}}{Cr^{51}\text{/ml blood}}.$$

Since the $Br^{82}$/ml blood was observed, blood chloride of bone was calculated as follows:

$$\frac{Br^{82}\text{/ml blood} \times \text{ml blood/g bone}}{SA\|} = \text{meq blood Cl/g bone.}$$

$\|$ SA = $Br^{82}$/meq. Cl.

Exchangeability of bone chloride was calculated as follows:

$$\text{Exchangeability (\%)} = \text{SA bone/SA serum} \times 100.$$

*Results.* Table I shows the observations on chloride of cat bone. The observed chloride content increased with increasing water content and decreasing body weight as did the estimated interstitial chloride content. However, an excess of chloride, above that calculated to be present in the interstitial fluid was observed in all bones. It is of interest that this excess of 1.53 meq/100 g fat free dry bone remained remarkably constant. The contents of ash and organic substances were noted to vary inversely within narrow limits.

Table II shows the contribution to the observed bone water and chloride contents by water and chloride calculated to be in blood of the marrow free cat bones. The small numbers of data do not warrant the assumption that this contribution increased with increasing bone water content.

Exchangeability of cat bone chloride as determined with $Br^{82}$ is plotted against water content of bones in Fig. 2. The exchangeability was as low as 47% in bone with lowest water content (11.7 g %) and rose rapidly with increasing water content until at a level of approximately 15 g % it had reached 95%. The value of 100% indicates that the specific activity of serum was equal to that of bone, and variation around this value is evidence of the level of agreement of measurements of chloride by titration and using $Br^{82}$. A difference of 7% was reached when exchangeability was at its maximum indicating a close agreement.

Table III illustrates observations on bone chloride in rats. Though there was an increase in observed chloride and estimated interstitial chloride in these animals with increasing water content, the excess chloride was not constant, and present only in animals under 150 days of age. The value for excess chloride of 1.76 meq/100 g in weanlings was

TABLE I. Excess Chloride of Cat Bone (11 Animals).

| Body wt (kg) | Water (g %)* | Ash (g %)* | Organic (g %)* | ECF chloride (meq/l) | Observed chloride (meq/100 g)* | Estimated interstitial chloride (meq/100 g)* | Excess chloride (meq/100 g)* |
|---|---|---|---|---|---|---|---|
| 2.0 | 11.7 | 69.4 | 30.6 | 129 | 2.91 | 1.51 | 1.40 |
| 3.8 | 12.4 | 67.1 | 32.9 | 126 | 2.82 | 1.56 | 1.26 |
| 2.4 | 13.3 | 67.9 | 32.1 | 123 | 3.30 | 1.64 | 1.66 |
| 2.9 | 13.8 | 66.2 | 33.8 | 126 | 3.49 | 1.74 | 1.75 |
| 3.8 | 13.9 | 70.1 | 30.0 | 127 | 3.35 | 1.77 | 1.58 |
| 2.6 | 15.5 | 68.5 | 31.5 | 123 | 3.51 | 1.90 | 1.61 |
| 1.0 | 20.8 | 68.3 | 31.7 | 123 | 4.15 | 2.55 | 1.60 |
| 1.1 | 24.6 | 65.9 | 34.1 | 124 | 4.39 | 3.05 | 1.34 |
| 1.4 | 25.1 | 64.0 | 36.0 | 134 | 4.71 | 3.36 | 1.35 |
| 1.0 | 27.1 | 66.3 | 33.7 | 122 | 4.90 | 3.31 | 1.59 |
| .9 | 34.7 | 63.2 | 36.8 | 129 | 6.20 | 4.47 | 1.73 |
| | | | | | Avg | | 1.53 |
| | | | | | Stand. dev. | | .17 |

* Reference bone weights are those for fat free dry bone.

TABLE II. Percentage of Observed Bone Water and Chloride as Blood Water and Chloride (Cat).

| Body wt (kg) | Bone | | | Blood water, % of total bone water (%)† | Blood chloride, % of total bone chloride (%) |
|---|---|---|---|---|---|
| | Water (g %)* | Blood (ml/100 g)* | Blood Cl (meq/100 g)* | | |
| 2.0 | 11.7 | .48 | .054 | 3.4 | 1.9 |
| 3.8 | 12.4 | .51 | .052 | 3.7 | 1.8 |
| 2.4 | 13.3 | .22 | .021 | 1.3 | .6 |
| 2.9 | 13.8 | .91 | .093 | 5.5 | 2.7 |
| 1.4 | 25.1 | 2.14 | .221 | 7.1 | 4.7 |

* Reference bone weights are those for fat-free dry bone.
† Calculated using observed water content of blood.

approximately the same as that found for cats.

*Discussion.* It is apparent that bone chloride content increased with increasing bone water content in both cats and rats. Subtraction of blood chloride from total chloride in cats does not eliminate this variation nor does subtraction of blood water from total water content eliminate variation in water content. The contribution of from 1.3% to 7.1% by blood water to total bone water is within the limits of 5-10% suggested by Neuman(5) for the amount present in the blood vessels, osteocytes, their lacunae and canaliculae.

It is also apparent that, in all but adult rats, there was more chloride present than would be estimated if all of the bone water had the same concentration of chloride as extracellular fluid. Since it has been estimated, by Eastoe and Eastoe(6), that 94% of the organic substance of cortical bone is collagen, and Nichols *et al.*(7) have found that dog tendon, which is comprised mostly of collagen, contains a chloride excess of 0.026 meq/g dry tissue, it is logical to postulate that the excess we observed was also located in collagen. However, in our experiment, only in cats did the ratio of excess chloride to organic substance remain constant. This ratio of 0.046 meq/g was the same as that found for weanling rats, but in this species, the ratio was not constant but decreased from the value of 0.047 meq/g in weanlings to zero in 150-day-old animals. It was also calculated that, since the ash and organic contents vary inversely in cat bone, ratio of excess chloride to ash also was constant at a value of 0.023 meq/g. Again in weanling rats this ratio was the same (0.028 meq/g) but decreased with increasing age in the same manner as the ratio of excess chloride to organic substances.

It was equally difficult to make any correlation of the ratios of excess chloride to water content since in cats the ratio decreased from 0.120 meq/g to 0.050 meq/g with an increase in water content from 12% to 35% while in rats the ratio increased from zero in bones containing 17% water to 0.034 meq/g in bones with 51% water.

The failure of any one of these ratios to remain constant in both species over the range of water concentrations and ages observed

TABLE III. Excess Chloride of Rat Bone.

| Age (days) | Water | Ash | Organic | ECF chloride (meq/l) | Observed chloride | Estimated interstitial chloride | Excess chloride |
|---|---|---|---|---|---|---|---|
| | (g %)* | | | | (meq/100 g)* | | |
| 157 (5) | 16.5 | 74.1 | 25.9 | 112 | 1.73 ± .12† | 1.84 | −.11 |
| 111 (2) | 22.7 | 72.4 | 27.6 | 110 | 2.67 | 2.49 | .18 |
| 98 (4) | 23.6 | 71.7 | 28.3 | 106 | 2.99 ± .08 | 2.49 | .50 |
| 92 (3) | 24.0 | 71.4 | 28.5 | 104 | 3.70 ± .31 | 2.51 | 1.19 |
| 82 (6) | 27.1 | 71.2 | 28.8 | 108 | 4.19 ± .39 | 2.93 | 1.26 |
| 28 (5) | 51.1 | 62.2 | 37.7 | 108 | 7.28 ± .58 | 5.52 | 1.76 |

* Reference bone weights are those for dry bone. † Stand. error. No. in parentheses refer to No. of rats analyzed.

makes it impossible to form, from these data, a satisfactory hypothesis concerning the location of excess chloride. It can only be stated that the fact that the exchangeability of the total bone chloride was less than 100% in presumably older cats with lower bone water concentrations suggests that the location of some of the chloride was less accessible in older animals. The very presence, however, of excess bone chloride makes the use of the chloride space as a measure of the extracellular fluid volume of bone unsatisfactory.

The accuracy of the chloride analysis is attested by the fact that measurements with $Br^{82}$, after maximum exchangeability was reached, were in close agreement with the titrometric analysis even though with $Br^{82}$, the only chemical measurement involved was plasma chloride. The observation that less than 5% of the observed bone chloride was present as blood chloride indicates that the samples were well cleaned of marrow and that this source of error was negligible.

*Summary.* 1) Observations on chloride content of bone of cats and rats revealed, in all but adult rats, that there was more chloride present than would be expected if the entire bone water content was extracellular fluid. This excess was constant at 1.53 meq/100 g of fat free dry bone in the cat over a wide range of water concentrations but, in rats, varied from a similar value in weanlings to the complete absence of an excess in 150-day-old animals. No satisfactory hypothesis could be made concerning the location of this fraction of the total bone chloride. 2) The accuracy of chloride analysis was attested by the maximum variation of 7% between titrometric determinations of chloride and determinations using $Br^{82}$. Studies with $Cr^{51}$ revealed that the blood content of well cleaned bone samples contributes less than 5% of the observed chloride and less than 10% of the observed water content.

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## X-Ray Photography of Human Cell Tumors in X-Ray and Cortisone-Treated Rats.* (24209)

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We wish to present a series of pictures, in reference to previously published data(1,2) showing the application of x-ray photography to the study of tumor development in treated weanling rats injected intraperitoneally with human cells. The cells implanted were from continuous cultures of HeLa, FL, and Detroit-6 strains. X-ray photography was a useful supplement to manual palpation for detection of solid tumors in the living animal. A permanent record of tumor growths in individual animals may be obtained in a series of x-ray photographs. The necessity for using a large number of animals for experimental purposes may be reduced by application of this method. This is particularly applicable when the wide variation in sizes of tumors, even under apparently uniform conditions is considered.

* Aided by Am. Cancer Soc. Grant.

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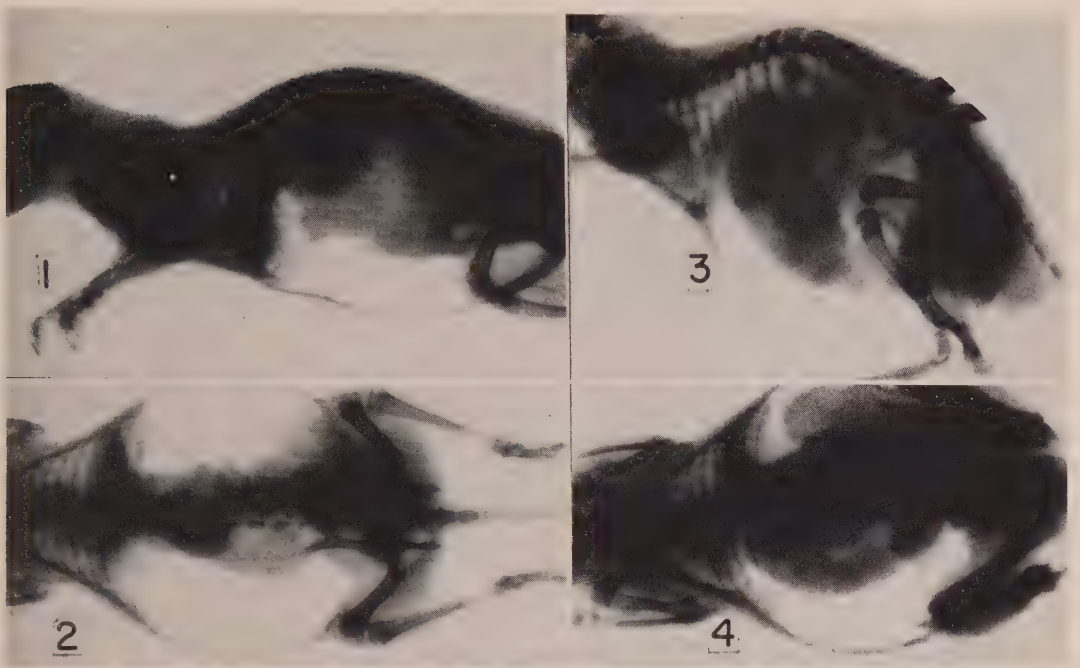


FIG. 1. Lateral view of rat (#549) without a tumor.
FIG. 2. Dorsal view of same rat.
FIG. 3. Lateral view of rat (#581) 19 days after inj. of HeLa cells. There is a medium sized tumor.
FIG. 4. Dorsal view of same rat 19 days after cell inj.

*Technic.* Rats to be photographed were starved overnight. The animals were anesthetized with ether, and 8-12 ml of air injected into 2 sides of the abdominal cavity. The x-ray equipment was the 100 KV Muller therapy unit, operated at 37 KV, 10 mA, with a 0.40 mm Al filter. Film target distance was 156 cm. Anesthetized animals were placed directly on the cassette and exposed in lateral view for 0.5 sec and in dorsal view for 0.5 sec. The 2 views were taken on the same x-ray film, half of plate covered with lead plate. Total dose of radiation under above conditions was 0.04 r for 2 exposures, as measured by Victoreen r-meter (Victoreen Instrument Co., Cleveland, O.). The film was the Eastman "Blue Brand," 5 x 7 inches, x-ray film designed for use with screen cassette. For further sharpness, a single screen technic was used. Screen below film was covered with opaque paper. Processing of film was standard for x-ray film.

*Results.* Fig. 1 to 10 show examples of x-ray pictures. No animals were lost due to this technic. Medium to large sized tumors were observed without difficulty, whereas

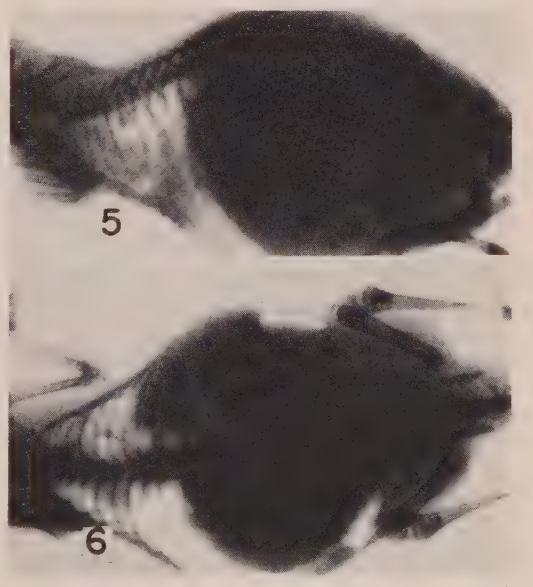


FIG. 5. Lateral view of rat (#581) 31 days after cell inj.
FIG. 6. Dorsal view of same rat 31 days after cell inj. Rat died the following day.

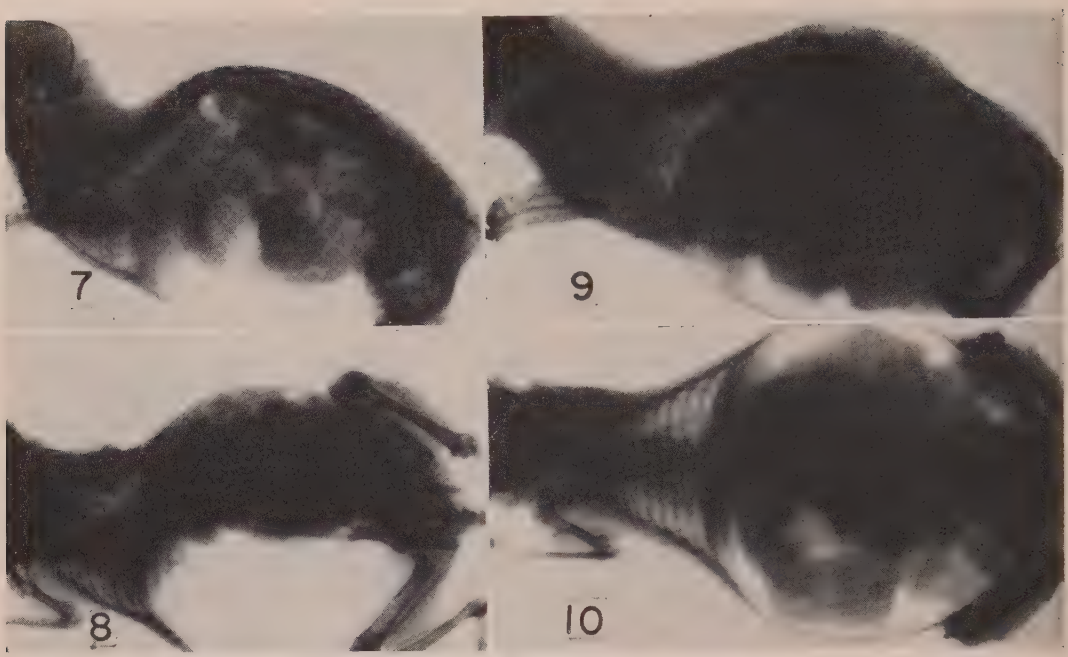


FIG. 7. Lateral view of another rat (#583) 19 days after inj. of HeLa cells. There is a small- to medium-sized tumor.

FIG. 8. Dorsal view of same rat 19 days after cell inj. Air was inj. only on one side. Picture shows that under such conditions determination of size and shape of the tumor is uncertain.

FIG. 9. Lateral view of same rat 25 days after cell inj. Tremendous increase in tumor size within the 6-day period.

FIG. 10. Dorsal view of same rat 25 days after cell inj.

identification of small tumors was unreliable. Detection of small tumors may require further refinement of technic.

Starvation of animals was an important step, since intestinal contents might mask the tumor. Air injected into the peritoneal cavity could be removed easily by needle.

*Discussion.* Our technic offers some advantages. Size, shape and to some extent, localization of tumors can be estimated repeatedly in the living animal. Exact measurement of size of animal can be recorded and compared to later stages of development. Animals can be used for various purposes during or after taking a series of x-ray photographs. We believe that the low dose of radiation did not affect the rat-tumor system.

*Summary.* Application of x-ray photography to the study of tumor development in treated rats injected with human cells is demonstrated.

We appreciate the assistance of Mr. A. Preston Hendrickson and Mrs. Lois Krueger.

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# Sodium Losing Material in Human Urine.* (24210)

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The purpose of this paper is to report evidence for existence of a material in urine of newborn infants and patients with congenital adrenal hyperplasia, with or without Addisonian symptoms, which induces sodium diuresis when injected into intact rats. Wilkins *et al.* described sodium diuresis following administration of ACTH to patients with congenital adrenal hyperplasia(1,2). Sodium diuresis of newborn infants given ACTH as well was reported in 1950(3). The possibility of the existence of an hormone causing sodium excretion was mentioned among other possible explanations. Prader reported that patients with congenital adrenal hyperplasia with Addisonian symptoms secreted aldosterone and that eventually secretion of aldosterone reached abnormally high levels(4). In 1955 urines from some premature infants in our study were assayed by Leutscher for aldosterone. He demonstrated excretion of 1 to 2 mg/day(5). These infants were receiving 5 to 10 meq sodium in each day's feedings. These and other infants were given ACTH and had a typical sodium diuresis. Urine from these premature infants after they were given ACTH was the source of the majority of material used in experiments herein described and of all the fractions previously reported to produce a diuresis of sodium(6).

*Methods.* Twenty-four hour urine collections were made from female premature infants 1 to 4 weeks old, given ACTH. Urine was collected from 2 newborn infants with congenital adrenal hyperplasia. One of these had Addisonian symptoms. The other, being treated with cortisone, was given ACTH. In addition, urine was obtained from 2 older patients with congenital adrenal hyperplasia, receiving cortisone. These patients were not given ACTH. One of these patients apparently had had Addisonian symptoms as an infant. Random 24 hour urines were collected from normal newborn infants. Urines were extracted with redistilled chloroform for the free steroid fraction. The residual urine was then adjusted to pH 4.5 and incubated 24 hours with 400 units of $\beta$ glucuronidase (Ketodase)/ml at a temperature of 36°C $\pm$ 1. Concentrated hydrochloric acid was added to urine to lower the pH to 1 and the hydrolyzed urine was reextracted with chloroform. The chloroform extracts were combined, washed with sodium hydroxide, then with distilled water. Chloroform extracts were passed through a florosil column prepared according to the method of Nelson and Samuels(7). The column eluates were dried under a stream of air at room temperature, dissolved in redistilled methanol and chromatographed on paper in the toluene-propylene glycol system of Zaffaroni(8). The portion of paper containing urinary extracts was then divided into several sections determined by rate of flow of standard steroids on the same paper. The first area extended from just below the origin at the level of standard compound E to a level just below dihydro compound E. Area 2 extended from this point to the level of standard tetrahydro compound A. Area 3 extended from this to the level of standard compound S. The paper from compound S until just before compound A was divided midway into the final 2 areas. The cut paper was then eluted with methanol. Eluates were dried under an air stream and portions were redissolved in 1 ml of olive oil. The final assay was carried out by injecting olive oil containing area 3 eluates into individual intact white male rats. Control rats were injected with the olive oil vehicle or olive oil containing 100 to 1000 mg of standard steroids or eluates of other paper chromatogram areas. The 24-hour urine excretions of sodium, potassium and creatinine were measured for in-

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† Markle Scholar in Medical Science.

TABLE I. Assay of Sodium Excreting Effect of Urinary Extracts.

| Days of assay | Area 3 eluate: Urine source | Rx | Days extract/rat | Inj. day Na excr., meq Na/mg cnne. | Control (mean ± S.D.), meq Na/mg cnne. | # S.D. by which exp. animals exceed control (mean) |
|---|---|---|---|---|---|---|
| 1 (not fed) | Premature infants | ACTH | 4 | .19 | .190 ± .024 ( 8)* | |
| | | " | 4 | .21 | | |
| 4 | *Idem* | " | 4 | .40 | .213 ± .044 (13) | >3 |
| | " | " | 4 | .33 | | >2.5 |
| 3 | " | " | 2 | .14 | .188 ± .043 (10) | |
| | " | " | 3 | .14 | | |
| 3 | " | " | 1 | .24 | .222 ± .046 ( 5) | |
| | " | " | 1 | .24 | | |
| 4 | " | " | 4 | .29 | .22 ± .049 (11) | <2 |
| 3 | " | " | 2 | .19 | .225 ± .035 (10) | |
| 4 | " "L" | " | 3 | .82 | .31 ± .066 (18) | >3 |
| 3 | " "A" | " | 2 | .55 | .30 ± .056 (18) | >3 |
| | | | 2 | .48 | | >3 |
| 4 | " "S" | " | 2 | .16 | .160 ± .035 (12) | |
| | | | 2 | .18 | | |
| 1 (not fed) | " "$H_1$" | " | 2 | .17 | .138 ± .043 (10) | |
| | " "H" | " | 1½ | .27 | | >3 |
| 2 | " "H" | " | ½ | .27 | .162 ± .036 (11) | =3 |
| | " "G" | " | 1 | .22 | | |
| | | | 3 | .26 | | >2.5 |
| 2 | " | " | 10 | .36 | .238 ± .040 ( 5) | >3 |
| (single 48-hr specimen) | " | " | 8 | .32 | | >2 |
| | | | 4 | .20 | | |
| 2 | " | " | 1½ | .16 | .182 ± .025 (10) | |
| | | | 3 | .26 | | >3 |
| | | " | 3 | .24 | | >3 |
| 4 | Full term newborn infants | 0 | 4 | .16 | .160 ± .035 (12) | |
| 3 | *Idem* | 0 | 8 | .22 | .225 ± .035 (10) | |
| 1 (not fed) | Newborn ♀ cong. adr. hyper. without Addisonian symptoms | ACTH | 1 | .52 | .186 ± .094 (10) | >3 |
| 4 | 1-mo-old ♂ cong. adr. hyper. with Addisonian symptoms | 0 | 1 | .34 | .217 ± .049 (12) | >2.5 |
| | | | <1 | .29 | | <2 |
| 2 | 10-yr-old ♀ cong. adr. hyper. without Addisonian symptoms | Cortisone | ½ | .22 | .210 ± .052 (17) | |
| 2 (not fed) | 7-yr-old ♂ cong. adr. hyper. without Addisonian symptoms | Cortisone (inadequate) 17 KS 1 | 2 | .24 | .150 ± .044 (16) | >2 |

* No. of control rats.

dividual rats. Standard steroids were compounds E, F, A, B, S and DOC as well as dihydro compound F, tetrahydro compound F, tetrahydro compound E, tetrahydro B and tetrahydro A, 17 hydroxy progesterone, 11$\alpha$ and 11$\beta$, 17 dihydroxy progesterone and 3-(3-oxo-17$\beta$-hydroxy-4-androsten-17$\alpha$-$\gamma$L) proprionic acid $\gamma$ lactone (SC-5523).‡ The last

‡ Kindly supplied by Dr. C. M. Kagawa, G. D. Searle and Co.

substance was shown by Kagawa *et al.* to antagonize aldosterone induced sodium retention in adrenalectomized rats(9). White male rats varied from 130 to 200 g but in any given experiment their weight did not vary more than 15 g on either side of the mean. Prior to the test assay the animals were kept in individual cages from 4 to 7 days, fed a standard rat chow and had access to distilled water *ad lib.* For the assay, rats were placed in metabolism cages over funnels draining urine into bottles containing thymol. The sides of collecting funnels were washed down with distilled water at the end of each day's collection. Rats were induced to void at beginning of assay and at end of each day's collection by application of an ether soaked sponge to the nose. Animals were given rat chow and distilled water during the test except in 4 assays where the rats were allowed only distilled water *ad lib.* to eliminate the possibility that urine was being contaminated by food particles. Sodium content of chow was 0.3%. Sodium and potassium measurements were made on internal standard flame photometer. Creatinine was measured by the method of Peters(10). Sodium and potassium excretions are reported per mg of urinary creatinine.

*Results.* None of the standard steroids, including SC-5233 in doses of 1 mg, nor any of the eluates from paper areas other than 3, produced any significant effect upon sodium and potassium output.

Table I lists the tests, source of urinary extract, number of days of assay, mean and standard deviation of control sodium excretion with number of control rats and sodium excretion on day of injection of individual rats tested with area 3 eluates. The pre- and post-injection excretions of sodium were similar for all rats and were equal to sodium excretion on day of injection of control rats.

The only material that significantly affected sodium excretion was the eluate of area 3, representing material found on paper chromatograms between levels of tetrahydro A and compound S. Thirty-three rats were injected with eluates of this area. This was variably associated with a slight increase in potassium excretion which was never statistically significant. Ten of these rats excreted amounts of sodium in their urine that were greater than the mean control excretions of sodium by more than 3 standard deviations. Three excreted sodium in excess of control means by 2.5 standard deviations and 2 rats had an equivocal sodium diuresis which exceeded their control means by only slightly more than 2 standard deviations.

Four eluates were divided into 2 unequal portions and each fraction injected into 1 rat. In each case the larger fraction provoked greater sodium diuresis. In 2 cases, each half of the original fraction gave an equivalent result when injected into individual rats.

The mean sodium to creatinine excretion on day of injection of 30 rats injected with area 3 eluates was 0.249 with a standard error of the mean of $\pm$ .016. The mean sodium to creatinine excretion of 280 control rat days combined was 0.192 $\pm$ .003. The difference between the 2 means is significant at the 99% level of confidence. (This calculation excludes 2 experiments carried out in 1956 when control sodium to creatinine excretion was significantly higher than subsequent control values.)

The actual creatinine excretions of rats given the extract of the area between tetrahydro A and compound S tended to be slightly but not significantly higher than the mean control excretion of creatinine. As may be seen in the chart, withholding food for the day of test did not seem to affect results other than to lower slightly the mean sodium to creatinine ratio.

A representative experiment is illustrated in Fig. 1. Two area 3 eluates were given to 2 individual rats. Ten control rats were injected with either pure olive oil or standard steroid material in olive oil. Sodium excretion is given for pre- and post-control days and day of injection of the 2 rats given area 3 eluate and mean sodium excretion is given for the 2 control days and day of injection for the 10 control rats. Dotted lines represent mean excretion for 30 control rat days $\pm$ 2 standard deviations on either side of the mean.

*Discussion.* The results of these tests dem-

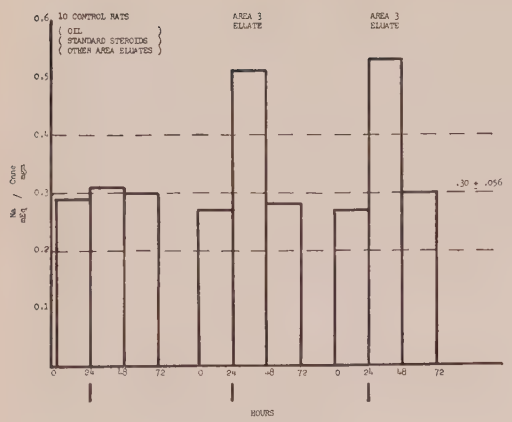


FIG. 1. Results of representative assay of area 3 eluates. Sodium excretion recorded per mg of urinary creatinine. First 3 bars represent mean excretion of 10 control rats for the 3 days of assay. Next 3 bars represent sodium excretion of an individual rat given a single area 3 eluate. Last 3 bars represent excretion of another individual rat so treated. Pips at bottom indicate time of inj. of either olive oil or standard steroid in olive oil in the case of 10 control rats and time of inj. of area 3 eluates in the 2 individual rats so treated. Dotted lines represent mean control excretion of sodium for the 30 control rat days ± 2 S.D. on either side of the mean.

onstrate the existence of material extractable from human urine under stated conditions which, when injected into intact rats, causes increased urinary sodium excretion. Evidence concerning the nature of this material will be published later.

There was no real attempt to quantitate the material used but when varying aliquots of the same material were injected into rats, the larger dose produced more sodium diuresis than the smaller and repeated injections of the same material gave reproducible results.

The material is more plentiful after ACTH administration and since it also resembles other urinary corticoids in its solubility, etc., it is suggested to us that it is a product of the adrenal cortex. However, this does not eliminate the possibility that the adrenal cortex is secreting commonly found steroids which are then metabolized along unusual lines by the liver, etc. Most of the material is found in the free fraction of urine of newborn infants. The significance of this is probably negligible since it has been shown that newborn infants conjugate steroids poorly and the majority of corticoids excreted are in the free fraction(11).

Our failure to demonstrate a sodium losing effect with each area 3 eluate may be the result of many possible factors acting singly or in combination. It may be explained by decreased efficiency of recovery of the material or decreased excretion of the material. The necessity of using intact rats complicates the experiments and the rats' own adrenals could be putting out antagonistic steroids under stress of injection, etc. The materials injected also may be contaminated with small amounts of active sodium retaining hormones. However, the principal sodium retaining hormone, aldosterone, should be well separated on the paper chromatographic systems used.

*Summary.* A substance has been obtained by chromatographic separation of extracts of urine obtained from premature infants and patients with congenital adrenal hyperplasia which when injected into intact rats produces an increase in urinary sodium excretion. The material is found more plentifully after ACTH administration to the patient but it has not been proven to be a secretory product of the adrenal cortex. It has minimal or no effect upon potassium excretion. It does not cause its effect by increasing glomerular filtration, at least as evidenced by creatinine excretion.

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# Comparative Ultrafiltration of Calcium and Strontium in Serum.*† (24211)

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Comparative metabolism of calcium and strontium in man and animals has become of importance with increase in fallout of $Sr^{90}$. Metabolic data are available from studies under various conditions for both human subjects(1-3) and animals(4,5) and on bone uptake(6-8). However, little is known about the various stages of passage of the 2 elements through membranes in the body. Once radiostrontium is absorbed and reaches the blood stream, filtration of strontium and of calcium through capillaries into interstitial fluid through the glomeruli, and through various membranes ensues. This filtration is of particular importance in assessing bone uptake and urinary excretion of these ions. The present study deals with *in vitro* filterability of the 2 ions in serum. It was expected that data obtained would permit an estimate of the relative binding of the 2 ions by plasma proteins.

*Materials and methods.* Blood was drawn by venipuncture from normals as well as from subjects with various pathologic conditions. Several large samples were also obtained uncitrated from the blood bank. In some cases, when special precautions were taken to keep the pH from rising, the blood was collected under oil and maintained under oil during centrifuging and pipetting. In addition, large samples of fresh beef blood were obtained from slaughterhouses. $Sr^{85}$ and $Ca^{45}$ were used as chlorides, the former without carrier, the latter with negligible amounts of stable Ca as carrier. The mixed isotopes were added in proportions of approximately 1 volume of isotope solution to 100 volumes of serum. Ultrafiltration was carried out in Visking cellophane casings, ¼″ diameter when inflated. Approximately 13″ lengths of casing were soaked in saline to separate the sides, and the saline then squeezed out. The outside of casing was wiped dry with gauze and a knot tied in one end. Approximately 1 ml of serum containing isotope solution was used to rinse out the rest of the saline. Five ml of fresh serum were then introduced and the other end tied off. The casing was now bent into U form and the 2 ends passed through the openings of a 2-hole rubber stopper and tied together. The rubber stopper was fitted into 40 ml centrifuge tube and the latter spun 15 minutes to an hour at approximately 600 g. From 0.5 to more than 1.0 ml of ultrafiltrate was obtained, depending on time of centrifuging. On analysis for N, several ultrafiltrates gave values equivalent to 0.2 g protein/100 ml or less, an amount corresponding to non-protein nitrogen. Samples were ultracentrifuged in duplicate and 0.1 ml samples pipetted. The same samples were used for $Sr^{85}$ counting and $Ca^{45}$ determination, high counts of both isotopes being used. Presence of $Sr^{85}$ slightly increased the $Ca^{45}$ count; the ratio of $Sr^{85}$ to $Ca^{45}$ was therefore kept low enough so that minor corrections were needed for both serum and ultrafiltrate, and net interference was negligible. In addition, several sera were ultrafiltered with $Ca^{45}$ alone to ensure that $Sr^{85}$ had no effect. Stable calcium was determined in serum by the method of Kramer and Tisdall as modified by Clark and Collip(9), phosphorus by the method of Fiske and SubbaRow(10). Protein was determined by the Kjeldahl method, a small correction being used for non-protein nitrogen. $Sr^{85}$ was counted in a well scintillation counter and $Ca^{45}$ was determined by precipitation with carrier as the calcium oxalate and counting in a thin window gas flow counter as previously described(11). Once stable Ca had been determined on serum, $Ca^{45}$ was used as a tracer, so that extent of ultrafiltration was followed by determining the $Ca^{45}$ in both serum and its ultrafiltrate. When stable Sr was added, $Sr^{85}$

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TABLE I. Ultrafiltration of $Sr^{85}$ and $Ca^{45}$ in Serum *In Vitro*.

| Diagnosis | % ultrafiltration 37.5°C $Sr^{85}$ | 37.5°C $Ca^{45}$ | 23°C $Sr^{85}$ | 23°C $Ca^{45}$ | Remarks |
|---|---|---|---|---|---|
| Carcinoma of uterus | 51 | 36 | 67 | 60 | 10.7 mg % Ca, 6.1 g % protein |
| " " breast | 60 | 45 | | | 10.6 6.8 |
| Cirrhosis, study I | 63 | 46 | 74 | 63 | 9.1 5.7 |
| " , study II (2 mo later) | 61 | 51 | | | |
| Carcinoma of breast | 59 | 39 | | | 9.9 6.3 |
| Multiple myeloma | 58 | 38 | | | 9.3 17.0 |
| Hypoparathyroidism | 62 | 38 | | | 8.5 7.6 |
| Normal (blood bank) | 67 | 39 | 72 | 44 | 11.5 6.6 |
| *Idem* | 57 | 40 | 61 | 43 | 10.6 6.8 |
| Saline control | | | 100 | 100 | pH 6.2 |
| *Idem* | 102 | 98 | | | pH 7.6, 10 mg % Ca, 3 mg % P |

was a tracer for it. pH was determined with a Beckman model G pH meter, and a minor correction made for effect of temperature by the method of Rosenthal(12). The pH was lowered when desired with a drop or 2 of HCl. Some experiments were carried out at room temperature, approximately 23°C, and others at 37.5°C in an incubator room.

*Results.* Table I shows results on a number of serum samples compared with those for saline solutions at different pH used as controls. Every serum tested showed greater ultrafiltration for $Sr^{85}$ than for $Ca^{45}$ at both room and body temperature. Filtration was also higher at 23°C than at 37.5°, but the differences were variable, and in normals inconsiderable for $Ca^{45}$. An experiment was performed with 2 sera to test whether these temperature variations were due to loss of $CO_2$ with a concomitant rise in pH at the higher temperature. A serum was first maintained at 37.5° and one portion centrifuged at that temperature; the other portion was then cooled to 23° and also centrifuged. There appeared to be a slight but definite temperature effect, $Sr^{85}$ filtration being 67 and 61% at 23° and 37.5° respectively, $Ca^{45}$ filtrations 50 and 43% respectively.

The effect of small variations of pH was tested by adding slight amounts of HCl to normal human and beef sera (Table II), and found to be small, in accordance with conclusions of McLean and Hastings(13) and other authors(14). The change in pH during ordinary handling of serum could therefore be expected to have a minor effect, slightly greater on $Sr^{85}$ filtration than on $Ca^{45}$.

Table III shows effect of addition of stable Sr. Amounts used did not appreciably alter either $Sr^{85}$ or $Ca^{45}$ filtration at 23°C, but seemed to increase $Sr^{85}$ filtration at 37.5° for serum A listed in Table III. Five mg% of stable Sr had no effect on serum B at pH of

TABLE II. Effect of pH on *In Vitro* Ultrafiltration of $Sr^{85}$ and $Ca^{45}$ in Serum at 37.5°C.

| pH | % $Sr^{85}$ filtered | % $Ca^{45}$ filtered |
|---|---|---|
| (a) Normal human serum, Ca 10.5 mg %, total protein 7.2 g/100 ml | | |
| 7.47 | 55 | 42 |
| 7.22 | 61 | 45 |
| 6.70 | 68 | 50 |
| (b) Beef serum, Ca 10.5 mg %, total protein 7.2 g/100 ml | | |
| 7.80 | 49 | 35 |
| 7.52 | 51 | 36 |
| 7.15 | 63 | 40 |
| 7.15 | 57 | 35 |
| 6.71 | 73 | 51 |
| 6.71 | 73 | 53 |

pH altered by addition of 1 or 2 drops of 2N HCl to 35 ml serum.

TABLE III. Effect of Added Stable Sr on Ultrafiltration or $Sr^{85}$ and $Ca^{45}$ in Serum *In Vitro*.

| Serum | Sr added, mg % | % ultrafiltered 37.5°C $Sr^{85}$ | 37.5°C $Ca^{45}$ | 23°C $Sr^{85}$ | 23°C $Ca^{45}$ | Remarks |
|---|---|---|---|---|---|---|
| A | 0 | 58 | 47 | 71 | 51 | |
| | 2 | 64 | 50 | 72 | 49 | |
| | 4 | 69 | 48 | 78 | 43 | |
| | 10 | 65 | 42 | 70 | 46 | |
| | 20 | 71 | 43 | 74 | 50 | |
| B | 0 | 56 | 43 | | | pH 7.78 |
| | 5 | 64 | 33 | | | 7.91 |
| | 0 | 69 | 46 | | | 6.92 |
| | 5 | 68 | 44 | | | 6.90 |

TABLE IV. Ultrafiltration of $Ca^{45}$ and Stable P in Presence and Absence of $Sr^{85}$.

| Serum | pH | % ultrafiltration $Sr^{85}$ | $Ca^{45}$ | P |
|---|---|---|---|---|
| 1 | 7.40 | 61 | 38 | 104 |
| | " | | 43 | 102 |
| 2 | 7.42 | 62 | 42 | 102 |
| | " | | 41 | 106 |
| 3 | 7.45 | 58 | 39 | 100 |
| | " | | 39 | 106 |

Blood drawn under oil from normal subjects. Temp. of ultrafiltration, 37.5°C.

6.8 or 6.9 but appeared to decrease filtration of $Ca^{45}$ at pH 7.91. This might have been due to formation of a slight amount of colloidal calcium phosphate at this high pH.

The possibility that colloidal calcium phosphate formation was responsible for low Ca filtrations was tested in experiments summarized in Table IV. Blood was collected and centrifuged under oil and inorganic phosphorus determined both on sera and ultrafiltrates. For all 3 sera, approximately 60% of $Sr^{85}$, 40% of $Ca^{45}$ and 100% of phosphorus were filtered. This indicated that no colloidal calcium phosphate had been formed, and a similar result was found by phosphorus determinations of serum and ultrafiltrate of the hyperparathyroid patient listed in Table I.

Presence of $Sr^{85}$ had no effect on relative $Ca^{45}$ counts, as shown by experiments on sera of Table IV carried out both with and without the Sr radioisotope. Length of centrifugation had no effect on ultrafilterability, the same percentage of both ions being filtered after 15 minutes or 1 hour, although the volume filtered in 15 minutes was 0.5 ml, that in 1 hour 1 ml.

*Discussion.* Our investigation was aimed to determine comparative ultrafilterability of strontium and calcium in serum, at different pHs, in the presence and absence of stable Sr and at room and body temperature. Under all conditions Sr filtered to a greater extent than Ca. The exact ratio of Ca to Sr filtration varied, but at 37.5°, values for $Sr^{85}$ in several sera of normals averaged approximately 60%, those for $Ca^{45}$ 40%. Similar values were found for sera of male patient with hypoparathyroidism and of a female patient with cancer of the breast. We may conclude that Ca is more completely bound to the proteins of serum than is Sr, more Sr than Ca remaining in a free form.

Values found for calcium are slightly lower than would be indicated by data of McLean and Hastings(13), who found approximately 50% ionized calcium. However, if our data for 23°C are used, and if per cent ultrafiltration is calculated on the basis of amount filtered in a given amount of water instead of serum, the results are in generally good agreement. On the basis of amounts filtered per given amount of water, our average values at 37.5° are approximately 65% for Sr and 43% for Ca, and at 23° approximately 70% and 50% respectively. Percentage of ionized calcium is not quite the same as percentage of filterable calcium, but McLean and Hastings showed that in general the factors that would make for different values would cancel out.

The data of Prasad and Flink(15) are also in general agreement with ours. They ascribe the high values obtained by some authors to the use of $CO_2$ to stabilize the pH, their own data indicating that use of $CO_2$ produces artifacts and gives abnormally high values. As our own experiments were carried out chiefly for comparison of Sr and Ca filtrations, we did not attempt to settle this question. We did examine the various factors making for experimental error, and by use of duplicate filtrations, as well as control experiments with saline, eliminated the possibility of radioactive contamination as a factor.

Lack of pH control has also been indicated as a reason for variability of results(16), but the effect of even fairly large changes in pH (Table II), although appreciable, would not account for all the differences. Even at a pH of 6.70, the Ca of blood in Table II was only 50% filterable.

Analytical methods used for Ca have also been examined. The present method for determination of $Ca^{45}$ has a reproducibility of approximately $\pm$ 3%. It lacks the precision claimed for some of the stable calcium methods in the literature, although the number of methods proposed indicates that these claims are rarely substantiated subsequently. It has the great advantage, however, that if a

reasonably high concentration of $Ca^{45}$ is used, the precision is just as good for ultrafiltrate as for serum, and there is unlikely to be a systematic error in analysis applicable to one and not to the other. As shown by McLean and Hastings(13), the ionic and protein-bound Ca in serum are in dynamic equilibrium, so that the $Ca^{45}$ in serum and ultrafiltrate is a measure of relative values of stable Ca. The point was also checked experimentally by allowing several sera to stand for different lengths of time after addition of the radioisotope. No differences were found. Apparently, therefore, use of $Ca^{45}$ as an analytical tool introduced no systematic error.

Whatever the factors contributing to different values for filtration of Ca and Sr, both ions were affected similarly, and the relatively greater filterability of Sr seems definitely established.

*Summary.* Sera of patients and normal individuals have been ultrafiltered through cellophane to determine relative filterability of Sr and Ca, using $Sr^{85}$ and $Ca^{45}$ as tracers. In every case, Sr was found to filter more completely, values for 37.5° averaging close to 60% for Sr, and 40% for Ca, calculated on the basis of total serum filtered. None of several variables tested affected relative filterability of the 2 ions. Neither addition of stable Sr nor slight changes in pH, such as might result from manipulation during the experiment, produced significant changes in filterability. There appeared to be a slight negative temperature effect, values for both isotopes being lower at 37.5°C than at 23°C, although the effect on $Ca^{45}$ was usually negligible. Results indicate that Ca is more completely bound to protein than is Sr, more Sr remaining in a free form.

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## *In vivo* Fixation of Antibodies in the Adrenal.* (24212)

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Bale and Spar(1) have shown by radiolabelled antibody technics that antibodies prepared in rabbits against rat kidney, when injected into rats, are fixed in the adrenals. This antibody may be an important factor in disease processes which have been attributed to action of anti-kidney antibodies. Its presence in high concentration in a particular site

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may affect the function of the gland. It is known that the hormones of the adrenal gland can stimulate more than one physiological process. Thus, some conditions observed in experimental glomerulonephritis(2) or experimental toxemia of pregnancy(3) may be attributed to anti-kidney antibodies present in this gland. Indeed, injection of rabbit anti-rat kidney serum has been reported by Lippman to cause an increase in adrenal size (2). However, he found no evidence of tissue damage in the gland on histological examination. The purpose of our report was to determine more precisely the site of the localized antibody in the adrenal gland by immunohistochemical fluorescent stain method. This technic is capable of greater resolution than is possible with radioiodine label methods although the latter are more sensitive(4) and permit detection of smaller amounts of fixed antibody.

*Materials and methods.* The rabbit anti-rat kidney serum was that used previously (4). The globulin fraction (G-anti-RK) was labelled with $I^{131}$ in accordance with our procedure(5). Free iodide was removed by dialysis against borate buffered saline rather than by the use of an ion exchange resin. Approximately one atom of iodine/molecule of protein was found after iodination. The globulin fraction of horse antiserum against rabbit globulin was that described previously (6). It was coupled with fluorescein isocyanate according to the procedure outlined by Coons and Kaplan(7), or with tetramethylrhodamine isocyanate as described by us(8). Prior to use, labelled horse antibody was treated with wet rat liver sediment to remove nonspecifically staining materials(9). All animals were sacrificed and perfused 18 hours after intravenous injection of antibody. Radioactivity measurements on perfused organs were made in a well-type scintillation counter when required and the tissue was then quickly frozen. Immunohistochemical staining was performed as follows: Tissue sections of thickness 5 $\mu$ were cut, placed on slides, and air dried. The slides were immersed in saline, the area around the tissue wiped dry and the sections stained with the fluorescein or rhodamine labelled antibody. Incubation was carried out at room temperature for 1 hr and washing limited to 10 minutes in buffered saline with constant agitation. The tissues were mounted in glycerol and observations made under a Reichert fluorescence microscope.

*Results.* Concentration of G-anti-RK which must be present in the adrenals to be detected by the fluorescein antibody was determined as follows: Varying quantities of $I^{131}$ labelled G-anti-RK were injected intravenously into rats. Adrenals and kidneys were assayed for radioactivity by counting, and for presence of rabbit antibody globulin by the fluorescein staining technic. Animals injected with radioactive normal globulins (GNS) served as controls. When a dose of 6.9 mg of radiolabelled G-anti-RK was injected into a rat, fixation to the extent of 90 $\mu$g and 26 $\mu$g protein/g tissue was obtained for the adrenals† and kidneys, respectively. In a rat receiving 15 mg, there was localization to the extent of 115 and 98 $\mu$g/g of tissue for these organs. At these concentrations, staining of adrenals was readily observed. A control animal given 15 mg of radiolabelled GNS localized 20 $\mu$g/g of adrenals and 15 $\mu$g/g of kidneys. However, this was not detected by the fluorescein labelled antibody. The reason for this lack of staining is apparently due to diffuse distribution of the GNS in the organs rather than being localized and concentrated in specific sites as the G-anti-RK.

Two rats receiving 2.2 mg of G-anti-RK localized to the extent of 7.6 to 10.6 $\mu$g protein/g of adrenal and 6.8 to 6.9 $\mu$g protein/g of kidney by the radioactive assay but positive staining by the fluorescein procedure was observed only in the kidneys. Control animals given approximately the same dose of GNS intravenously showed no staining.

Although the average concentration of localized antibody was greater in the adrenal

† Localization in the adrenal expressed/g tissue must be considered to be a rough estimate because of abberations in weight of such a small organ (10-20 mg) due to perfusion and drying during the weighing process.

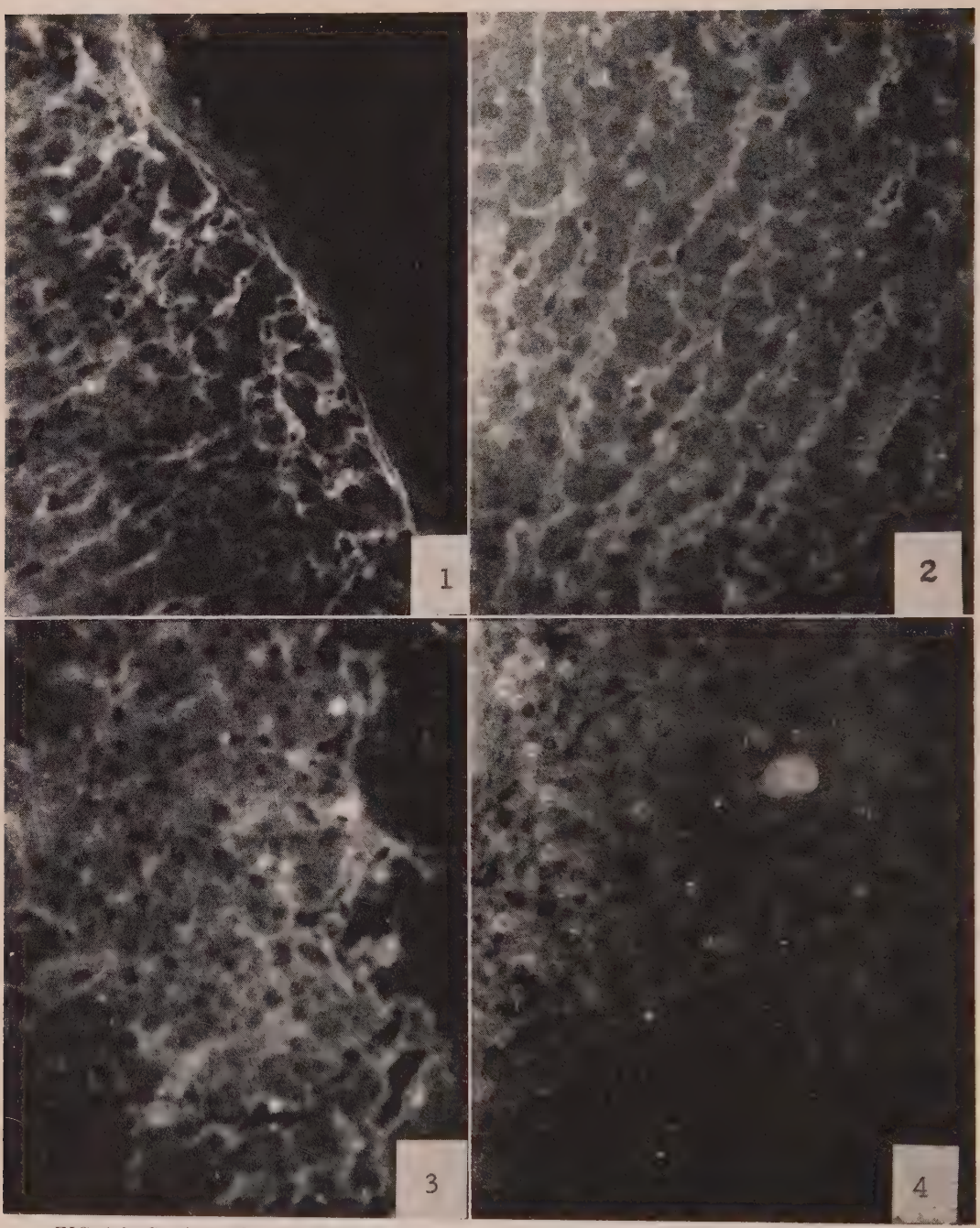


FIG. 1-3. Sections of adrenal from rats inj. with 15 mg G-anti-RK and stained with fluorescein labelled horse anti-rabbit globulin antibody.

FIG. 1. Presence of rabbit anti-rat kidney antibody is seen in the sinusoids of the zona glomerulosa. No fixation in capsule or parenchymal cells seen.

FIG. 2. Fixation is seen in sinusoids of the zona fasciculata.

FIG. 3. Fixation is seen in sinusoids of the zona reticularis up to the medullar boundary. No fixation of G-anti-RK was observed in the medulla.

FIG. 4. Adrenal from rat receiving 15 mg GNS and stained as above. No fixation was demonstrable in this area of the zona fasciculata.

than in the kidney, actual concentration of antibody globulin at site of fixation was less since antibody could be detected only in kidney at the lowest amounts injected.

After initial use of $I^{131}$ labelled protein to determine limits of detection, studies dealing with the description of the histological site of the localized antibody were carried out with unlabelled G-anti-RK. Control rats were injected with the same amount of globulin from pooled normal rabbit sera.

Fixation of antibody takes place just inside the capsule along the sinusoids when small amounts, 4 to 7 mg, of G-anti-RK are administered. When a dosage of 15 mg or greater is administered, the sinusoids throughout the organ show the fixed antibody. This gives the appearance that the antibody is being cleared as it progresses through the adrenal from the capsule inward. The cells near the capsule are more advantageously situated with respect to fresh blood; in these areas fixation seems to take place first with appreciable clearance of antibody from the blood (Fig. 1). Only when these sites become inefficient due to incipient saturation does localization further inside take place.

The fixation was not limited to the zona reticularis alone as reported by Bale and Spar (1) for antibodies prepared against rat adrenal as observed by radioautography. We found that the antibodies studied here (prepared against rat kidney) were seen throughout the cortex, *i.e.*, also in the zona glomerulosa (Fig. 1) and the zona fasciculata (Fig. 2). No fixation was observed in the capsule or the large polygonal parenchymal cells.

An important observation concerns the lack of fixation in the medulla. Staining occurs up to the medullary boundary but no staining is discernible beyond the boundary (Fig. 3). The absence of antibody in the medulla cannot be due to lack of blood vessels since there is a rich capillary network which surrounds the cords and clumps of cells in this region.

The cells lining the capillaries of the smaller vessels in the medulla are endothelial, whereas those lining the sinusoids in the cortex are littoreal cells of the macrophage system, much like those lining the sinusoids of the liver and hypophysis(10). This difference in the lining cells may possibly explain the lack of fixation in the medulla of injected anti-kidney antibody.

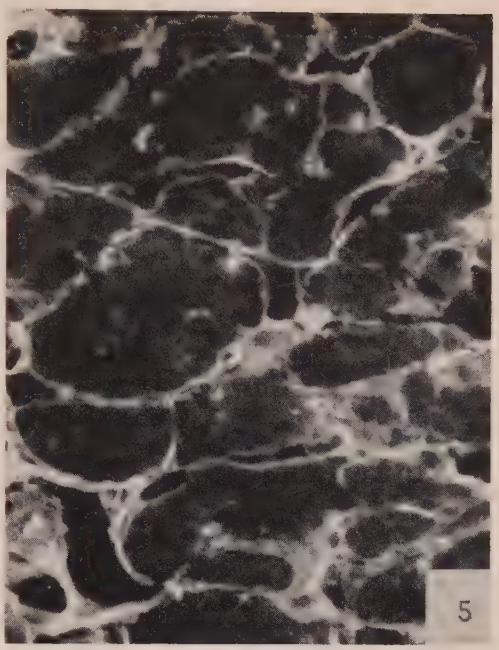


FIG. 5. Adrenal section from rat treated *in vitro* with G-anti-RK and stained as above. Antibody fixed in medulla and in the reticular connective tissue elements surrounding the large parenchymal cells.

The medulla does contain substances which can react with anti-kidney antibody when brought into contact with it as when sections of adrenals from uninjected rats are treated with G-anti-RK and developed with fluorescein labelled horse anti-rabbit globulin antibody. In addition to the staining of the sinusoids of the cortex, staining occurs in the capsule and in the reticular elements surrounding the cells of the medulla (Fig. 5). Cruickshank and Hill(11) found similar staining of the adrenal by application of fluorescein labelled G-anti-RK directly. The *in vitro* and *in vivo* situations are different since in the latter case the antibody may not be able to make contact with the antigen.

A similar condition has been shown to exist for the kidney and lung. Anti-kidney antibody is fixed *in vivo* to a detectable concentration only in the glomeruli although it is known to contain antibodies capable of re-

acting with other antigens of the kidney(11, 12). Lung also contains antigens capable of reacting with kidney localizing antibody but does not fix them efficiently *in vivo*(13).

The enlargement of the adrenal gland of rats injected with nephrotoxic serum although showing no histological damage(2) is probably a manifestation of increased permeability of the cortical sinusoids in this organ caused by the extensive localization of the heterologous antibody in these sites.

*Summary.* The site of fixation in the adrenal gland of rat kidney antibody administered *in vivo* has been demonstrated by the immunohistochemical technic to be along the vessels and sinusoids of the cortex. There is no fixation in the medulla although the medulla does contain antigen capable of reacting with the antibody *in vitro*. The possible derangement of adrenal function may be due to effect of the anti-kidney antibodies.

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## Rapid Method of Metabolically Characterizing Individual Tumors.* (24213)

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Characterization of neoplasms as to class by usual methods of pathology is well known to have but limited value in selection of chemotherapeutic agents for treatment of individual tumors. Previous studies have shown that the chromatographic spectrum of metabolic constituents of crude animal tissues often yielded highly specific information allowing for laboratory differentiation of disease processes appearing the same with the light microscope(1). With this in mind a variety of glial tumors together with a number of tumors metastatic to the brain were chromatographed and the patterns obtained after spraying with ninhydrin recorded. In addition to this, fresh tumor tissues were studied individually in relation to amino acids which they took out of a synthetic media of known composition. In this report only the tumors morphologically classed as glioblastoma multiforme will be considered.

*Materials and methods.* Tumors were obtained in the fresh within a few minutes after surgical extirpation. Representative portions of the tumor were sent to the neuropathology laboratory for the usual pathologic study and diagnosis. Other representative parts were cut into small fragments, using a dissecting microscope to eliminate necrotic tissue as far as possible, squeezed into sheets of Whatman No. 1 chromatographic paper, dried in a hood at room temperature and chromatographed in

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butanol acid water (40 : 10 : 50) solvent system using the ascending method. The details of this technic have been previously described (1). After 18 to 20 hours the developed chromatograms were dried and sprayed with 0.4% ninhydrin and acetone, heated in an incubator at 70° for 10 to 15 minutes, and the patterns derived in the individual tumor studied and recorded photographically. Other representative pieces of fresh tumor were cut into tiny fragments, again special care being taken to eliminate visible necrotic tissue with the dissecting microscope, and about 50 mg of the minced tumor tissue placed in 3 cc of Difco culture medium TC 199 and thereafter incubated for 24 hours at 37°C. Following incubation, the cells were sedimented by centrifugation and supernatant taken for analysis. Two to 3 parallel tests were run on each tumor. 0.01 cc of media from each run of individual tumors were put alternately along the base line of Whatman No. 1 chromatographic paper sheet intermixed with these was 0.01 cc of media never exposed to tumor cells. After drying, the sheets were developed in butanol acetic acid solvent for 18 to 20 hours, dried and sprayed with 0.4% ninhydrin and acetone, as above. With this technic the following amino acids may be generally separated: glutamic acid, phenylalanine, tyrosine, methionine, alanine, histidine, leucine, threonine, lysine and cystine. The strips may be judged visually for very rough quantitative estimation of amount of amino acids present, as judged from density and size of individual spots or may be much more sensitively judged through examination in an analytical densitometer. This latter method was mainly used. The results of the study of the media after exposure to the tumor cells provided a further control as to any influence of necrosis of the cells upon the pattern of uptakes. In occasional flasks new ninhydrin positive substances not present in the original media were seen. In such instances the uptakes of certain of the amino acids by the individual tumor were hidden. In some of these the flask contained a larger amount of amino acids than was present in the original media. Such flasks did not figure in the results. As might be expected there was some variability in the amounts of individual amino acids taken up by the individual tumor from flask to flask. In every instance however, those taken up in the largest amounts always repeated though the amino acids taken up in proportionately smaller amounts might be missed. The same result generally held in those tumors whose uptakes were measured on separate occasions. An example of this is the comparison of tumor 11 with tumor 34 which was taken from the same individual with an interval of several months between. In studies of 3 tumors in the group of 12 being reported, an amount of ethionine equivalent to the amount of methionine originally in the media was added to test whether the influence of the analogue might exert a measurable effect on amino acid uptakes of tissue under study.

*Results.* In tumors numbered 1 through 11, individual tumors were tested only as to whether they took up amino acids at all, rather than quantitatively. Furthermore, studies of these tumors did not include a parallel test of the ninhydrin positive pattern of the constituents of the crude tumor. The remainder of the tumors were studied quantitatively and in relation to the ninhydrin pattern of crude tissue. In tumors numbered 17 through 39 the percentages indicate the amount of individual amino acids removed from the media by tumor cells during 24 hour incubation. Though it is evident that these various glioblastomas have much in common, particularly as to utilization of glutamic acid, tyrosine, phenylalanine, leucine and methionine, individual variation from tumor to tumor as to relative proportions of various amino acids used, and in some instances utilization of certain different amino acids, for example alanine, histidine and lysine, appears the rule rather than the exception. Similar relationships were found upon comparison of patterns obtained in chromatographing the whole tumor tissue. In Fig. 1 a rather remarkable similarity is seen between the pattern in tumor 19 on comparison of tumor 35, though certain differences are still evident. That the amino acid uptakes of these 2 tu-

TABLE I. Glioblastoma.

| Tumor | Glutamic | Phenyl-alanine | Tyrosine | Methio-nine | Alanine | Histidine | Leucine | Lysine | Cystine | Ethionine effect |
|---|---|---|---|---|---|---|---|---|---|---|
| | (% uptake) | | | | | | | | | |
| 1 | × | × | × | | | | | inc. | | |
| 3 | × | | | | | | | | | |
| 5 | × | | | | | | | | | |
| 10 | × | × | × | × | × | × | | | | |
| 11 | × | × | | | | | | | | |
| 17 | 40 | 20 | 50 | | 50 | | 30 | | | |
| 19 | 20 | | 15 | 20 | | | 10 | | | ×××× |
| 22 | 50 | | | | | | | | | |
| 30 | | | 50 | 30 | | | 20 | | | ××× |
| 34 | 50 | 20 | 20 | | | 30 | | | | none |
| 35 | 30 | 15 | 20 | 20 | | | 10 | | | |
| 39 | 15 | | | | | | 15 | 30 | | |

mors also had great similarity is noteworthy (Table I). The chromatographic pattern obtained from tissue of tumor 17 and tumor 34 showed a much more considerable difference both in relation to each other and to tumors 19 and 35 (Fig. 1). It is pertinent that the relative proportion of various amino acids taken up by tumors 17 and 34 was quite different, and further that one of these tumors, 17, took large amounts of alanine from the media, and 34, large amounts of histidine (Table I). Both these findings are in contrast to the general run of glioblastomas. The pattern found in tissues of tumor 39 was also highly individual (Fig. 1). This might have been expected from its relative amino acid uptakes as well as its utilization of a large amount of lysine. In tumor No. 19 the presence of ethionine completely prevented uptake of any of these amino acids as measured by this system. In tumor No. 30 the uptake was almost entirely blocked. In contrast in tumor No. 34 the ethionine had no effect whatever upon the uptake of various amino acids. It is noteworthy that the 2 tumors, 19 and 30, had similarities both as to amino acid uptake and as to their tissue chromatographic pattern. It is also pertinent that tumors 19 and 30 utilized methionine quite extensively in contrast to tumor 34 in which no utilization was discernible. In view of the fact that ethionine is the metabolic antagonist to methionine and, in these 3 tumors, the ones in which methionine was used were the ones in which ethionine was effective, it would seem worthwhile to find whether the specific antagonist of amino acids utilized by an individual tumor might be a chemotherapeutic agent of choice to treat the individual tumor.

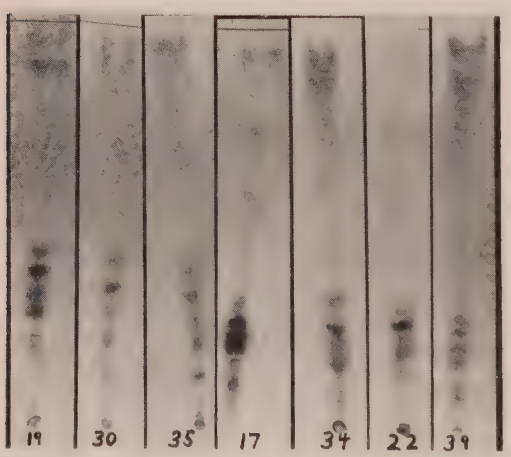


FIG. 1. Comparison of chromatographic patterns of several glioblastomas as shown with ninhydrin following development in butanol acetic acid water solvent system.

It would appear from these results that the combined studies of individual tumors by means of technics of classic pathology, study of the chromatographic pattern of individual tumors, and study of metabolic requirements of the same individual tumors will yield useful information leading both to understanding of the metabolic difference between normal and neoplastic cells and lead as well to a more effective utilization of chemotherapeutic agents.

*Summary.* A rapid method for biochemically characterizing individual neoplasms particularly in relation to amino acid content and requirements is described. It is suggested

that use of this method will facilitate the more accurate choice of chemotherapeutic agents in treatment of individual tumors even of the same morphologic class, and perhaps be an effective means of rapidly screening metabolic antagonists as to their effect upon individual tumors.

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## Effect of Beta-Carotene and Cortisone on Vaginal Epithelium of Vitamin A Deficient Rats.* (24214)

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Conversion of beta-carotene to Vit. A is partially influenced by tocopherols, Vit. $B_{12}$, antibiotics, phosphates, insulin and thyroxin (1). Recently Clark and Colburn(2), utilizing biochemical analyses, found that administration of cortisone to rats on stock laboratory diet or Vit. A free diet caused rapid reduction in quantity of Vit. A in liver and kidney. They further demonstrated that only when large amounts of beta-carotene were administered to cortisone-treated rats was there adequate conversion of beta-carotene to Vit. A and deposition of Vit. A in liver and kidney. They suggested that conversion of beta-carotene to Vit. A is impaired in cortisone-treated rats.

The results obtained by Clark and Colburn (2) seem to indicate that cortisone-treated animals would eventually become Vit. A deficient and exhibit manifestations of the deficiency. However, it is also possible that blocking of the conversion of beta-carotene to Vit. A by cortisone may result in quantitative reduction of the Vit. A stored in body depots, and may not produce pathological changes in organs sensitive to Vit. A deficiency. With this question in mind, and by utilizing cornification of the vaginal epithelium of the rat as criterion of Vit. A deficiency(3), the present investigation was undertaken to determine whether beta-carotene in the presence of cortisone treatment would cause cornification of vaginal epithelium of Vit. A deficient rats to disappear.

*Methods.* Fifty-one rats of Wistar strain were placed on a Vit. A free diet‡ on day 20 of life. Daily weighings and vaginal smears were begun 3 weeks after initial feeding of Vit. A free diet.

When the Vit. A deficient animals exhibited vaginal cornification continuously for 3 to 4 days, they were subjected to the following procedure. In Group I (17 animals), the rats received daily either 6, 12, 24, 48, or 480 $\mu$g of beta-carotene§ orally for 10 days. Rats of Group II (6 animals) received 2.5 mg of cortisone acetate subcutaneously/day for 10 days but received no beta-carotene. Animals in Group III (19 animals) for 10 days received daily subcutaneous injections of 2.5 mg of cortisone acetate and in addition either 12, 24, 48, or 480 $\mu$g of beta-carotene orally. Rats in Group IV (9 animals) received 2.5 mg of cortisone acetate subcutaneously/day for 3 days prior to dual treatment of cortisone and beta-carotene daily for the next 10 days. Dosages of beta-carotene used for animals of Group IV were 12, 24, or 48 $\mu$g.

At the end of prescribed treatment, the animals were sacrificed and the vagina of each animal removed and fixed in Zenker's solution. After fixation the tissue was dehydrated, imbedded in paraffin, and sectioned at

* Supported by grant from Nat. Vit. Found.

† Student Part-time Research Fellow of U.S.P.H.S., Division of Nat. Inst. of Health.

‡ Vit. A Test Diet U.S.P., Nutritional Biochemicals Corp., Cleveland, O.

§ The beta-carotene was supplied through the courtesy of Hoffmann-LaRoche, Nutley, N. J.

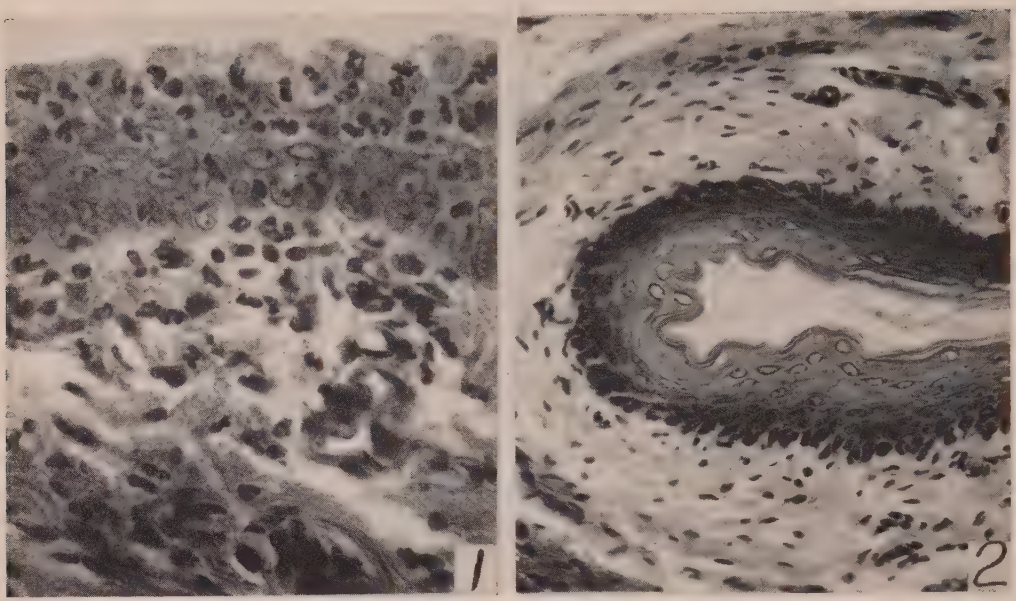

FIG. 1. Photomicrograph of portion of vagina of vit. A deficient animal treated with beta-carotene. Cornified epithelium of vit. A deficiency is replaced by columnar and cuboidal shaped cells. Neutrophiles are also present throughout. Sections of vagina of vit. A deficient rats treated with beta-carotene and cortisone are similar to this photomicrograph. Weigert's hematoxylin and eosin (×390).

FIG. 2. Photomicrograph of portion of vagina of vit. A deficient animal treated with cortisone. Vaginal epithelium is stratified squamous keratinized and like that of vit. A deficient animals. Weigert's hematoxylin and eosin (×230).

8 $\mu$ and stained with Weigert's hematoxylin and eosin.

*Results.* The first manifestation of Vit. A deficiency was abnormal cornification of the vagina. This appeared from 4 to 5 weeks after beginning of the feeding of Vit. A free diet. Beginning with the fifth week the animals steadily decreased in weight. Since cornification of the vagina and decrease in body weight are well known symptoms of Vit. A deficiency, further consideration of those changes will not be presented.

After the third day of treatment with beta-carotene alone (Group I), vaginal smears of all animals contained only nucleated cells and neutrophiles. The keratin layer of the stratified epithelium of the vagina was replaced by columnar and cuboidal shaped cells (Fig. 1). The new cells at surface of stratified epithelium had large nuclei and contained many intracytoplasmic vacuoles. Also, neutrophiles were present among cells of the stratified epithelium and in the lumina of the vaginae. The results demonstrate that beta-carotene suppresses abnormal cornification of the vagina in Vit. A deficient rats.

In animals that received 2.5 mg cortisone acetate alone (Group II), abnormal cornified epithelium of the vagina continued unchanged (Fig. 2). This demonstrated that cortisone acetate alone had no effect on cornified epithelium of Vit. A deficient rats. The changes observed in vaginal epithelium of rats which received the hormone and beta-carotene concurrently (Group III), were similar to those observed in the animals that received beta-carotene alone, thereby demonstrating that beta-carotene does influence the epithelium of the vagina in the presence of cortisone. The alterations observed in the vaginae of animals in Group IV, receiving cortisone alone for 3 days prior to combined treatment of beta-carotene and cortisone, were similar to those observed in rats that received beta-carotene alone or beta-carotene and cortisone.

*Discussion.* The cuboidal and columnar shaped cells that replaced the keratin layer of the stratified epithelium of the vagina in

beta-carotene treated animals are similar to those which occur in the vagina of the rat after topical application of Vit. A(4,5). Our results, following beta-carotene treatment are similar to those of Sherwood, Brend and Roper(6) who observed nucleated cells in vaginal smears of rats following oral treatment with beta-carotene.

Since rats receiving cortisone alone gave no indication of alterations in the vaginal smear, it seems clear that changes observed in the beta - carotene or beta - carotene - cortisone treated animals must have been the result of conversion of beta-carotene to Vit. A.

No estrogenic activity follows local or subcutaneous treatment with cortisone(7,8), therefore, persistence of cornified epithelium in the vagina of deficient animals treated with cortisone was due to Vit. A deficiency, and not to treatment with the hormone. Under our conditions, therefore, cortisone had no demonstrable effect on conversion of beta-carotene to Vit. A.

The present observations do not completely contradict the work of Clark and Colburn(2), who found a reduction in the quantity of Vit. A stored in liver and kidneys. The possibility of cortisone having an effect on storing of Vit. A, and not on conversion of beta-carotene to Vit. A, must be recognized.

*Summary.* Abnormally cornified epithelium of the vagina induced by Vit. A deficiency disappeared when beta-carotene alone or beta-carotene and cortisone was administered. Cortisone alone did not modify the cornified epithelium of the vagina. The results demonstrate, therefore, that cortisone has no demonstrable effect on conversion of beta-carotene to Vit. A.

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## Failure of Blood Glucose Levels to Reflect Hepatic Glycogenolysis; Experiences with Glucagon.* (24215)

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It is common practice in some experimental situations to measure hepatic glycogenolysis indirectly through changes in blood glucose levels. Accurate blood glucose determinations can be performed rapidly and blood samples can be obtained conveniently at any desired interval. Direct determination of liver glycogen, on the other hand, requires liver biopsy or sacrifice of the experimental animal, and it is not practicable to perform repeated determinations on the same subject during an acute experiment. On the basis of serial blood sugar determinations, it has been stated that glucagon is inactive when administered subcutaneously(1,2). In the course of our studies of glycogen metabolism, we have in several instances drawn erroneous conclusions from such indirect evidence. It is the purpose of this communication to call attention to the hazards of such indirect "measurement" of glycogenolysis and to emphasize the necessity of performing glycogen analyses.

*Methods.* Normal and adrenodemedullated male Sprague-Dawley rats, weighing 200 to 260 g, were used. Blood glucose was

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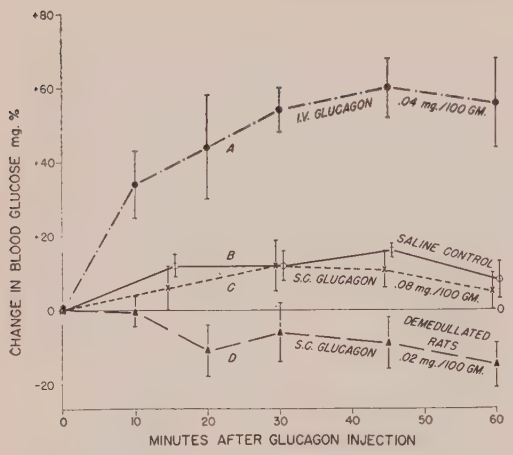


FIG. 1. Blood glucose curves after glucagon inj., illustrating range of variation encountered. Vertical lines represent stand. error.

determined by the Nelson(3) method, using tail blood. Liver specimens were obtained under nembutal anesthesia. Glycogen was determined by the method of Good, Kramer and Somogyi(4) and expressed in terms of glucose (Nelson method). Glucagon was supplied by the Eli Lilly Laboratories as a powder, lot No. 258-234B-54-2. This material has negligible insulin activity. It was dissolved in slightly alkaline distilled water, then diluted with physiological saline. Rats were fasted 24 hours, then given a standard feeding of 0.60-0.75 g of glucose/100 g body weight, by stomach tube. Four and one-half to 5 hours were allowed for deposition of liver glycogen. Glucagon was then injected intravenously, intraperitoneally, or subcutaneously, in doses ranging from 5 to 80 $\mu$g/100 g body weight. Controls received slightly alkaline physiological saline. Blood samples for glucose determinations were obtained at 10- to 15-minute intervals. Animals were sacrificed one hour after glucagon injection, and glycogen determination was performed.

*Results.* Considerable variation was observed in blood glucose curves after injection of glucagon. Rises in blood glucose of 24 to 104 mg % were observed, after intravenous administration of 20 to 40 $\mu$g of glucagon/100 g to normal animals. When similar doses were injected intraperitoneally or subcutaneously, distinct, but smaller rises in blood glucose levels usually resulted. In some experiments, however, no significant rise could be demonstrated. In the adrenodemedullated rats, even intravenous glucagon injection produced only slight increases, and subcutaneous administration regularly failed to raise blood glucose level. Degree of variation observed in this study is shown in Fig. 1, which presents data from 3 experiments, involving 17 rats.

The effect of glucagon on liver glycogen, on the other hand, was consistent and reproducible. Marked glycogenolysis was induced in every one of 104 animals injected with glucagon. The results of these glycogen determinations are presented in Table I. The values corresponding to the blood glucose curves in Fig. 1 are identified by the same code letters. At all dose levels investigated, the glycogen depletion at one hour after subcutaneous injection was at least as intense as that observed after other routes of administration.

*Discussion.* Blood glucose level reflects the operation of many well known metabolic processes which require no discussion here. Of importance, however, is the fact that the en-

TABLE I. Liver Glycogen One Hour after Glucagon Injection.

| Glucagon dose and route of administration | | Liver glycogen ± S.E. (%) | |
|---|---|---|---|
| **Normal rats** | | | |
| Control, saline | | 3.49 | .12 |
| .005 mg/100 g Intraper. | | 1.10 | .15 |
| *Idem* Subcut. | | .40 | .03 |
| Control, saline | | 2.71 | .12 |
| .01 mg/100 g Intraper. | | .80 | .11 |
| *Idem* Subcut. | | .76 | .14 |
| Control, saline | | 2.51 | .18 |
| .02 mg/100 g Intraper. | | .10 | .03 |
| *Idem* Subcut. | | .10 | .06 |
| Control, saline | | 1.98 | .18 |
| .02 mg/100 g Intrav. | | .50 | .30 |
| .04 mg/100 g " | (A) | .06 | .01 |
| Control, saline | | 2.78 | .23 |
| .04 mg/100 g Intraper. | | .63 | .38 |
| *Idem* Subcut. | | .39 | .15 |
| Control, saline | (B) | 2.51 | .51 |
| .08 mg/100 g Subcut. | (C) | .13 | .12 |
| **Adrenodemedullated rats** | | | |
| Control, saline | | 2.92 | .22 |
| .02 mg/100 g Subcut. | (D) | .24 | .09 |
| Control, saline | | 1.89 | .21 |
| .04 mg/100 g Intraper. | | .16 | .07 |
| *Idem* Subcut. | | .13 | .09 |

tire hepatic glycogen content represents only a small store of glucose, when compared to capacity of the glucose disposal mechanisms. Calculations from oxygen consumption data (5) and standard metabolic tables indicate that at a respiratory quotient of 0.85, the rat can completely oxidize within one hour the glucose represented by a 3% liver glycogen level. The data of Stetten and Boxer(6) indicate that this amount of glucose is converted into fatty acids within 2 hours by rats on a high carbohydrate diet. A barely detectable rise in muscle glycogen—0.1%, can account for one-third of this glucose reserve. Thus, relatively small increases in activity of these disposal mechanisms, operating together, can rapidly remove from the blood the glucose produced by relatively intense hepatic glycogenolysis. It is noteworthy that a single hormone, insulin, accelerates all 3 of these mechanisms. Other physiologic processes which may be of importance in this regard include gluconeogenesis, the rate of which can also vary greatly.

When glucagon is administered intravenously, hepatic glycogenolysis is initiated very rapidly and the activity of the glucose disposal mechanisms does not increase sufficiently to prevent a transient hyperglycemia. Subcutaneous injection, on the other hand, may fail to induce a rise in blood glucose, although glycogenolysis is of the same order of magnitude. Presumably, in such experiments, the onset of glycogenolysis is not as rapid, and glucose disposal mechanisms are stimulated in time to handle the increased glucose load. Even with intravenous injection, however, the rise in blood glucose accounts for only a fraction of the glucose liberated from the liver.

The adrenodemedullated rats showed little or no rise in blood glucose after glucagon administration. Such animals are more sensitive to insulin than are normal animals(7). The earlier stimulation of glucose disposal mechanisms in these rats is consistent with the hypothesis that endogenous insulin release is responsible for the prevention of hyperglycemia during glucagon glycogenolysis.

The experience reported here is not unique for glucagon. Similar findings have been observed with a glycogenolytic liver extract(8) and, more recently, with the synthetic agent, mercaptoethylamine (unpublished). Other examples of discrepancy between changes in liver glycogen and blood glucose are better known. Liver glycogen declines concurrently with a falling blood glucose after insulin administration(9). Epinephrine administered to a fasted animal causes hyperglycemia and liver glycogen deposition(10). Thus, in any experiment where stimulation of insulin or epinephrine secretion is possible, great caution should be exercised in drawing inferences regarding hepatic glycogenolysis or glycogenesis from the blood glucose values. Direct determination of liver glycogen must be performed, at least in representative animals.

*Summary.* 1) Glucagon is as active a glycogenolytic agent when administered subcutaneously as it is by intraperitoneal or intravenous route. However increases in blood glucose may not be observed following subcutaneous administration of glucagon, even though liver glycogen is almost completely mobilized. In adrenodemedullated animals, this is the rule. 2) Total liver glycogen store of the rat is relatively small, compared to the capacity of glucose disposal mechanisms. Changes in rates of the major glucose disposal reactions can completely mask the anticipated effect of hepatic glycogenolysis on blood glucose concentration. Such changes may be induced by fluctuations of endogenous insulin secretion.

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# Relation of Structure of Synthetic Muscarines and Muscarones to Their Pharmacological Action.* (24216)

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Muscarine was first extracted from *Amanita muscaria* by Schmiedeberg and Koppe (1). The chemical structure proposed by Kögl *et al.*(2) was shown to be in error when Eugster and Waser(3) and Eugster(4) isolated pure, crystalline muscarine, determined the empirical formula, $C_9H_{20}O_2NCl$, and obtained evidence for presence of a tetrahydrofurane ring. Kögl *et al.*(5) established by X-ray crystallography the structure of muscarine as a stereoisomeric form of 2-methyl-5-dimethylamino-methyl - tetrahydrofurane - 3-ol methochloride. As muscarine belongs to a group of tetrahydrofurane compounds with 3 asymmetric carbon atoms, 8 stereoisomeric forms are possible. The first syntheses of muscarine resulted in mixtures of stereoisomers(6). A stereospecific synthesis of L-(+) muscarine was reported by Hardegger and Lohse(7). More recently, the synthesis of all racemic stereoisomers of muscarine has been accomplished(8). The present study aims to correlate muscarinic action to stereostructure by comparing pharmacologically (a) natural and synthetic L-(+) muscarine (b) L-(+) muscarine with other muscarine isomers (c) these muscarines with some closely related muscarone compounds.

*Materials.* The following compounds were studied: L-(+) muscarine chloride, natural, Fig. 1,A(3); L-(+) muscarine chloride, synthetic(9); D-(−) muscarine chloride(9); dl-muscarine chloride(10); and dl-muscarine iodide(10); dl-epi-muscarine iodide, Fig. 1,B (10); dl-allo-muscarine iodide, Fig. 1,C(11); dl-epiallo-muscarine iodide, Fig. 1,D(8); (+) - muscarone iodide ($[\alpha]_D$ + 11.2°, water) and (−) muscarone iodide ($[\alpha]_D$ - 11.5°, water), Fig. 1,E, prepared through resolution of dl-normuscarone(10) with the aid of di-p-toluyl-tartaric acids and quaternization of the (+) and (−) normuscarones (Eugster *et al.*, unpublished); dl-muscarone iodide(10); dl-allo-muscarone iodide, Fig. 1,F(11); dl-4,5-dehydro muscarone chloride, Fig. 1,G(10).

*Methods. Pelvic nerve-bladder preparation of the dog.* The bladder of female dogs under pentobarbital anesthesia was cannulated and connected with water manometer. The pelvic nerve was electrically stimulated with condenser discharge pulses (frequency 40-200/sec. duration of pulses 1 msec., voltage 1-10 V.) every 10 sec. for 1/5 second duration to avoid spontaneous movements of bladder. The compounds were administered through the cannulated left iliac artery into the abdominal aorta employing of each compound 2 doses differing by a ratio of 1:2. Each compound was tested in 3 to 7 experiments. Mean equiactive dose ratios relative to muscarine chloride, and their standard deviations were calculated; doses were expressed as number of molecules of a compound rather than on a weight basis. The effective dose range of muscarine was between 0.025-0.4 μg/kg. *Isolated rabbit ileum.* Segments of rabbit ileum were suspended in tissue bath according to the method of Magnus. 2 x 2 point assays were performed in 4 to 7 experiments with each compound. Mean equiactive dose ratios relative to muscarine and their standard deviations were calculated. Muscarine was effective in concentrations of 0.005 to 0.02 μg/ml. *Miosis in mice.* The method of Shingh Grewal(12) using albino mice was modified in that mecamylamine (5 mg/kg, intraperitoneally) instead of atropine was given to dilate the pupil. The muscarines were given intraperitoneally, simultaneously with mecamylamine, in 3 logarithmically spaced doses to groups of 6 mice each. The pupil size was determined after 15 and 30 minutes. The potencies of compounds were expressed by equiactive dose ratios and their

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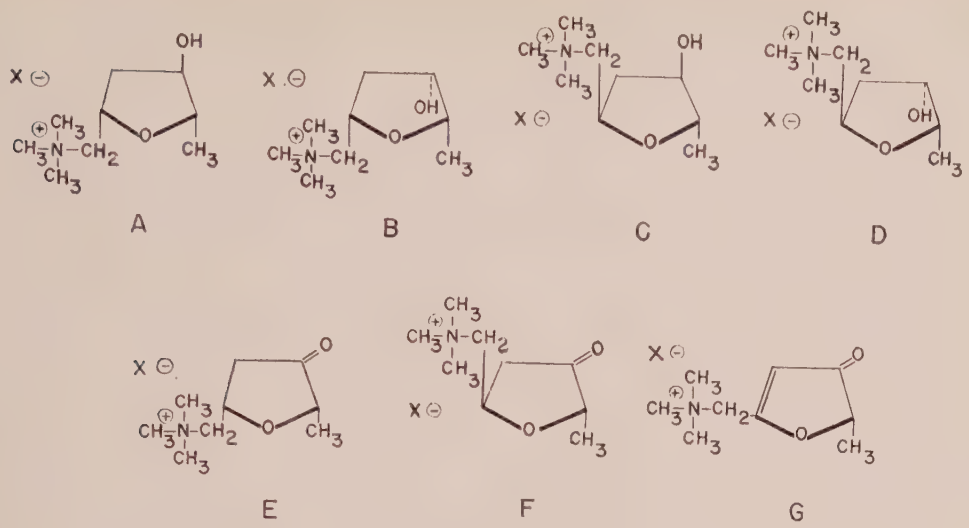


FIG. 1. Steric structures of muscarine and muscarone homologues.

fiducial limits relative to muscarine chloride. *Frog rectus abdominis muscle.* The rectus muscle of *Rana pipiens* was suspended in frog Ringer solution at room temperature. Contractures were recorded isometrically *via* a strain gage and a polygraph. Since muscarine-like compounds have a slower onset of action than acetylcholine, each drug was in contact with the tissue for 4 to 5 minutes. The compounds were given at 2 or 3 dose levels, and the approximative equiactive doses relative to acetylcholine chloride were calculated on the basis of 2 to 4 experiments.

*Results.* Table I presents the data obtained on postganglionic parasympathetic action of muscarine compounds on 3 types of smooth muscle. Relative potency of the compounds determined in dog bladder preparations closely agrees with that obtained in tests on the isolated ileum and on the pupil. In all 3 tests synthetic muscarine was equal in potency to natural muscarine.

D-(−) muscarine has been found at least 200-800 times less potent than the L-(+) form. It has been reported to have less than 5% of the potency of the L-(+) form when tested on the cat's blood pressure(9), and to be inactive on the isolated frog heart(13). Racemic muscarine was 2.5 times less active than the L-(+) form indicating also that the D-(−) form is inactive.

The allo, epi, and epiallo isomers are several hundred times less potent than racemic muscarine; dl-epimuscarine being the least potent isomer.

Table I also presents data on the action of close congeners of muscarine in which a keto group replaces the hydroxyl group. These muscarones are more active than the corresponding muscarines. Racemic muscarone iodide is about 8 times more potent than dl-muscarine iodide. The optically active (−) form is more active, the (+) form only slightly less active than the racemate. In contrast to the findings on dl-allo-muscarine, dl-allo-muscarone is only slightly less potent than dl-muscarone. The unsaturated 4,5-dehydro muscarone is about twice as potent as dl-muscarine.

The results of tests on the frog rectus muscle (Table I) show that the nicotinic potency of the alkaloids of the muscarine group is negligible; it is at least a few hundred times less than that of acetylcholine. In contrast, the compounds of the muscarone group cause appreciable neuromuscular stimulation. They surpass acetylcholine in potency.

*Discussion.* The experiments demonstrate a definite stereospecificity of the action of the muscarines on postganglionic parasympathetic effector sites: epi, allo, epiallo muscarines possess only a negligible fraction of the

TABLE I. Comparative Potency of Muscarine and Muscarone Homologues.

| Compounds | No. of molecules equivalent to 1 molecule of natural muscarine chloride | | | | | | | | | No. of molecules equivalent to 1 molecule of acetylcholine |
|---|---|---|---|---|---|---|---|---|---|---|
| | Dog bladder | | | Isolated rabbit ileum | | | Mouse miosis | | | Frog rectus abdominis muscle |
| L-(+) muscarine chloride, natural | 1.0 | | | 1.0 | | | 1.0 | | | >400 (3)† |
| *Idem*, synthetic | .9 | .042* | (6)† | .95 | .055* | (7)† | .86 | ( .58–1.21)‡ | | >200 (2) |
| D-(−) muscarine chloride | ~800 | | | ~400 | | | >200 | | | |
| dl-muscarine chloride | 2.55 | .43 | (4) | 2.4 | .24 | (4) | 2.3 | (1.5 –4.0 ) | | >400 (2) |
| dl-muscarine iodide | 2.77 | .34 | (6) | 3.1 | .24 | (6) | 1.6 | ( .8 –2.5 ) | | >600 (2) |
| dl-epi-muscarine iodide | 1700 | | (3) | >700 | | (3) | >600 | | | " (2) |
| dl-allo-muscarine iodide | 500 | | (3) | 460 | 60 | (4) | >600 | | | " (2) |
| dl-epiallo-muscarine iodide | 365 | 111 | (4) | 660 | 145 | (4) | >140 | | | >280 (2) |
| + Muscarone iodide | .52 | .59 | (4) | .45 | .07 | (6) | .32 | ( .15– .58) | | .5 (2) |
| − Muscarone iodide | .17 | .019 | (4) | .19 | .019 | (6) | .19 | ( .12– .28) | | .3 (2) |
| dl-muscarone iodide | .35 | .009 | (4) | .4 | .05 | (7) | .2 | ( .11– .3 ) | | .5 (3) |
| dl-allo-muscarone iodide | .77 | .05 | (4) | .84 | .24 | (6) | .97 | ( .7 –1.2 ) | | .3 (2) |
| dl-4,5-dehydro-muscarone chloride | 1.62 | .33 | (6) | 1.5 | .25 | (6) | 3.0 | (2.3 –3.9 ) | | .5 (4) |

* ± stand. dev. of mean equiactive dose ratio. † No. in parentheses = No. of experiments.
‡ Fiducial limits.

potency of the dl-muscarine. Furthermore, the enantiomers of muscarine differ greatly in their potency; the potency seems to reside almost exclusively in the L-(+) form. All stereoisomers of muscarine are devoid of significant action on the skeletal muscle.

On the contrary, a stereospecificity of the pharmacological action cannot be demonstrated with the enantiomers and the allo isomer of dl-muscarone; these muscarones did not differ greatly among each other in their action on smooth muscle. There were, however, potent stimulators of skeletal muscle, at least equal to acetylcholine in causing contracture of the frog's rectus muscle. Thus, dl-muscarone was 8 times were potent than dl-muscarine on smooth muscle; on skeletal muscle it was more potent than acetylcholine.

Substitution of the hydroxyl group of muscarine by a keto-oxygen also involves a reduction from 3 asymmetric to 2 asymmetric carbon atoms. Thus, it cannot be stated that either one of these changes is responsible for the difference in stereospecificity and in nicotinic action between muscarines and muscarones. It is tempting to speculate that the pronounced nicotinic potency of the muscarones results from introduction of the keto group *per se;* the muscarones with the keto oxygen-ether group bear a greater structural resemblance to acetylcholine than does muscarine with the hydroxyl-ether group. It is interesting to note that 4,5-dehydromuscarone possesses less muscarinic but somewhat greater nicotinic potency than dl-muscarone.

*Summary.* 1. Comparison of the potency of muscarine and synthetic congeners on smooth muscle (gut, bladder, iris) and on skeletal muscle demonstrated equal potency of synthetic and natural L-(+) muscarine. 2. The potency of muscarine is stereospecific;

D-(−) muscarine and all racemic stereoisomers (epi, allo, epiallo) possess only a fraction of the potency of dl-muscarine. 3. Keto analogues of muscarine (muscarones) are more potent than muscarine in stimulating smooth muscle; in addition, they possess, on frog skeletal muscle, remarkable nicotinic activity of which the muscarines are almost devoid. 4. The potency of the muscarones is not stereospecific. Both + and − muscarones are more potent than L-(+) muscarine, and dl-allo muscarone is only slightly less potent than dl-muscarone.

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## Alterations in Serum Proteins of Hibernating Hamsters.* (24217)

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In view of the manifold metabolic adjustments which accompany natural hibernation (1,2,7) it is not unreasonable for one to expect an alteration in the partitions of serum proteins during this state. That certain such changes may occur in at least the hibernating hedgehog (*Erinaceus europeueus*) was shown by Biörck *et al.*(3) whose data also indicated that there was no change in total serum protein level during hibernation in spite of a marked increase in hematocrit. The primary aim of our work was to determine what changes take place in serum proteins upon assumption of hibernation by the golden hamster (*Mesocricetus auratus*). We also have attempted to follow these changes in small series of cold exposed hamsters prior to onset of hibernal sleep and following spontaneous arousal therefrom.

*Materials and methods.* Other than control hamsters, maintained at room temperature, all animals were kept in a temperature controlled room at 5 ± 0.5°C. Experimental animals were divided into 3 groups: *Group 1* was exposed to the cold for various intervals of time, but had not hibernated; *Group 2* had hibernated continuously for 2 days; *Group 3* had been awake in the cold for one and 2 days after *spontaneous* arousal from hibernation. All animals were killed by cervical fracture. Blood for serum electrophoresis and hematocrit determinations was obtained directly from right ventricle after exposure of heart. Serum was analyzed for total protein by micro biuret method with pure bovine serum albumin serving as standard(8). Duplicate 0.1 ml samples of the serum were then subjected to paper electrophoresis using a Durrum type cell (Spinco commercial model R). After 20 hours at 5 ma. in a 0.05 M veronal buffer the electrophoretic strips were dried, stained with brom phenol blue and analyzed using the

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TABLE I. Serum Proteins during Cold Exposure and Hibernation. Concentration expressed as g/100 ml.

| | | Total protein | Albumin | *Alpha*$_1$-globulin | *Alpha*$_2$-globulin | *Beta*-globulin | *Gamma*-globulin | Hematocrit |
|---|---|---|---|---|---|---|---|---|
| Controls | (10) | 7.30 .12* | 2.68 .09 | 1.26 .11 | 1.02 .10 | 1.00 .11 | 1.36 .12 | 46.3 1.78 |
| 1 day cold exposed | (3) | 6.13 .59 | 2.43 .09 | .84 .08 | .74 .18 | 1.43 .06 | .711 .08 | 46.4 3.44 |
| 2 days " " | (3) | 7.58 .46 | 2.45 .23 | 1.09 .05 | 1.42 .23 | 1.18 .16 | 1.115 .33 | 50.1 1.62 |
| 5 " " " | (3) | 7.50 .73 | 2.90 .16 | 1.01 .06 | .00 .10 | 1.55 .41 | 1.31 .32 | 51.8 1.89 |
| 10 " " " | (6) | 7.42 .24 | 2.58 .20 | 1.33 .06 | .82 .05 | 1.42 .26 | 1.17 .20 | |
| 2 mo " " | (5) | 7.54 .12 | 2.43 .10 | 1.36 .07 | 6.81 .08 | 1.76 .09 | 1.20 .12 | |
| Hibernating | (14) | 8.87 .38 | 3.40 .07 | 1.38 .05 | 1.02 .08 | 1.74 .13 | 1.00 .10 | 62.2 .97 |
| 1 day after arousal | (5) | 6.56 .33 | 2.39 .08 | 1.06 .05 | .75 .07 | 1.14 .24 | 1.21 .24 | 50.9 .52 |
| 2 days " " | (3) | 6.90 .11 | 2.19 .14 | .876 .09 | 1.50 .22 | 1.49 .09 | .911 .09 | 50.8 1.01 |

* Stand. error. No. of animals used are contained in parentheses.

Beckman Analytrol Scanner. Concentrations of the various protein fractions were calculated from the relative percentage of each fraction and the total serum protein content (Table I).

*Results.* The most obvious alterations in serum protein levels were related to the hemoconcentration that accompanied cold exposure and hibernation. Hematocrits of hibernating animals were about 34% higher than those of controls. Concentrations of total serum protein and serum albumin showed increases of 22 and 27% respectively ($P < 0.01$ in all cases. While albumin, the major serum protein, increased in concentration, the total circulating quantity of the fraction was probably not altered, since the rise was approximately equal to that of the hematocrit. Upon arousal, there occurred a general decrease in hematocrit and proteins approximating control or pre-hibernation levels. *Alpha*-globulins did not show a significant increase during hibernation but, considered on the basis of percentage of total protein, an actual decrease in total circulating amount was indicated. The reality of this decrease is questionable since it is well known that complete separation of these fractions from albumin by paper electrophoresis is usually poor, at best.

Of greater interest was the observation that the *beta*-globulins showed an increase prior to and during hibernation which was significantly greater than could be accounted for on the basis of simple concentration of serum proteins.

In contrast to the disproportionate increase in the *beta*-globulins was the marked decline which took place in *gamma*-globulin during torpidity.

*Discussion.* From data presented here, it would appear that changes in serum proteins of the hibernating hamster were brought about both by a simple hemoconcentration and through operation of other, as yet unexplained, factors. The hemoconcentration, as indicated by the more-or-less commensurate increases in hematocrit, total serum protein and serum albumin concentrations, confirms those data obtained by others studying other hibernating species(1,3,4). In partial conflict with our observations are those of Biörck *et al.*(3) on the hedgehog. These investigators found no change in total serum protein concentration during hibernation, in spite of the fact that the reported hematocrits rose significantly. Their results also differ from ours in that they found a decrease in *beta*-globulins whereas we found a significant increase.

Of the changes which occurred in serum proteins, and which could not be explained on the basis of hemoconcentration, the fall in *gamma*-globulin was the most striking. Whether this is due to a lack of stimuli for immunological reactions as suggested by Biörck(3) or to some metabolic peculiarity is not possible to ascertain at present.

Alterations in the circulating quantity of *alpha* and *beta* fractions raise the question of nutritive adaptations since *alpha*$_1$-globulin and *beta*$_1$-globulin are especially associated with blood lipids(5).

Upon examining the data, the possibility that the various fractions are differentially stored or removed from active circulation, or in the case of the *beta*-globulins released into

circulation from auxiliary stores, during hibernal sleep might be considered. In contrast to the hibernating hamster, the rabbit displays a decreased concentration of serum *beta*-globulins and an elevation of *gamma*-globulin during simple cold exposure(6).

*Summary.* 1) Alterations in various fractions of serum proteins and hematocrits were studied in hibernating and non-hibernating hamsters. 2) During hibernation the hematocrit, serum albumin and *beta*-globulins increased to a significant degree. The increase in *beta*-globulins was too large to be accounted for on the basis of simple hemoconcentration. Since concentrations of neither $alpha_1$ nor $alpha_2$-globulin increased, the data were interpreted to mean that total circulating quantity of these globulins decreased. A marked decline in concentration of *gamma*-globulin occurred.

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## Metabolism of Human Leukocytes *in vitro* V. Inhibition by Human Serum of Formate and Glycine Incorporation.*† (24218)

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Human leukocytes can be isolated, incubated *in vitro,* and observed to incorporate labeled precursor compounds into cellular protein, nucleic acid, and acid-soluble components at rates characteristic of cell type and maturity(4). Incorporation of formate(4) and glycine(1) has been previously shown to be significantly more rapid in Krebs-Ringer-Bicarbonate-Glucose media than in normal human serum, suggesting that serum may contain inhibitors or dilutors of metabolic processes in which these precursors are involved. These findings may suggest the presence of a substance or substances in normal circulation which regulate metabolic processes in leukocytes. The physiological and clinical implications of such regulators might prove to be of great interest. Further studies of the effect of serum on formate and glycine incorporation into normal and leukemic leukocytes *in vitro,* therefore, were undertaken.

*Materials and methods. Separation, incubation, and fractionation of leukocytes.* Leukocytes were collected in citrate-fibrinogen, washed in saline, suspended in 1 ml of Krebs-Ringer-Bicarbonate-Glucose (KRBG)§ me-

* This work supported by Grants from Nat. Inst. of Health, P.H.S. Blood specimens were obtained from patients at Illinois Research and Educational, Cook Co., West Side Veterans Admin., and Presbyterian Hospitals, Chicago, and at Veterans Admin. Hospital, Hines, Ill. We wish to thank the many physicians from these institutions who aided us.

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§ The following abbreviations have been used in this paper: KRBG = Krebs-Ringer-Bicarbonate supplemented with 3 mg/ml glucose; KRB = Krebs-Ringer-Bicarbonate; GPF = gross protein fraction; ASF = acid soluble fraction; CLL = chronic lymphocytic leukemia; CGL = chronic granulocytic leukemia; and AL = acute leukemia.

TABLE I. Effect of Normal Human Serum and Serum Derivatives on Formate Incorporation into Intact Human Leukocytes.

| Incubated cells (0.5 to 1.0 × $10^8$ cells/beaker) | (Final vol = 2.2 ml/beaker) | Gross protein fraction, % inhibitions of formate uptake* |
|---|---|---|
| (A) Chronic lymphocytic leukemia | Krebs Ringer bicarbonate | 0 † |
| | Fresh normal serum | 37 § |
| | Serum ultrafiltrate‖ | 40 § |
| | Boiled serum ultrafiltrate¶ | 30 § |
| (B) Chronic granulocytic leukemia | Krebs Ringer bicarbonate | 0 † |
| | Serum ultrafiltrate‖ | 64 § |
| | Ether extract of S.U.F.** | 6 † |
| | Ether residue of S.U.F.** | 78 § |
| (C) Acute granulocytic leukemia | Krebs Ringer bicarbonate | 0 † |
| | Fresh normal serum | 30 § |
| | Serum ultrafiltrate‖ | 30 § |
| | Dialysate†† | 17 ‡ |
| | Residue†† | 0 † |

* 100 (1 − incorporation in test media/incorporation in K.R.B.).

† $p < .01$ in comparison with fresh normal serum or serum ultrafiltrate.

‡ $p < .05$ in comparison with K.R.B. of same experiment using pooled replicate variance for experiment.

§ $p < .01$ for same.

‖ Fresh normal serum ultrafiltrated at 4°C through cellophane membrane under pressure.

¶ Serum ultrafiltrate kept at 100°C for 30 min. and reconstituted to the original volume.

** Serum ultrafiltrate extracted with equal volumes of diethyl ether, the ether and aqueous layers brought to dryness and each reconstituted to original vol.

†† Fresh normal serum dialyzed against an equal vol of Krebs Ringer bicarbonate medium and the dialysate tested. Residue was then dialyzed against a large vol of water and the residue tested.

dium(3) to which was added either 1 ml of serum or serum ultrafiltrates or Krebs-Ringer-Bicarbonate (KRB) media(3) and 0.2 ml of a saline solution of formate-$C^{14}$ (.67 $\mu$M, 2.0 $\mu$C), or glycine-1-$C^{14}$ (1.41 $\mu$M, 2.0 $\mu$C). All incubations were carried out in triplicate incubations, usually for 4 hours. Each beaker contained potassium penicillin G and streptomycin sulfate in concentrations of 0.15 mg/ml each. Separation of the gross protein fraction and calculation of specific activity and per cent inhibition were performed as previously outlined(4).

*Results.* Table I summarizes the results of 3 typical experiments showing that the presence of fresh normal human serum decreased formate incorporation into the gross protein fraction (GPF) of various types of leukemic leukocytes by 30 to 64%. This effect is due to low molecular weight components of the serum since the activity passes through a cellophane membrane during dialysis or ultra-filtration. It is ether-insoluble and heat-stable.

The time course of formate incorporation into leukocyte GPF in the presence and absence of serum dialysate was found to be linear with time in both instances, but to be 30% slower in the presence of the serum dialysate (Fig. 1a). Fig. 1b shows incorporation of formate in a 4-hour incubation period as a function of this amount of normal serum in the incubation medium.

Table II shows that normal serum ultrafiltrate decreases incorporation of glycine as well as formate into the gross protein fraction of chronic granulocytic leukemia leukocytes. Studies with other protein and nucleic acid precursors will be required to determine whether the effects of serum are general or limited to formate and glycine.

Table III summarizes the data on the effect of normal human serum on formate uptake by normal and leukemic leukocytes. It is apparent from these data that all cell types are decreased by normal serum to approximately the same degree.

It was of interest to determine whether there are differences between the effect of serum from various normal and pathological

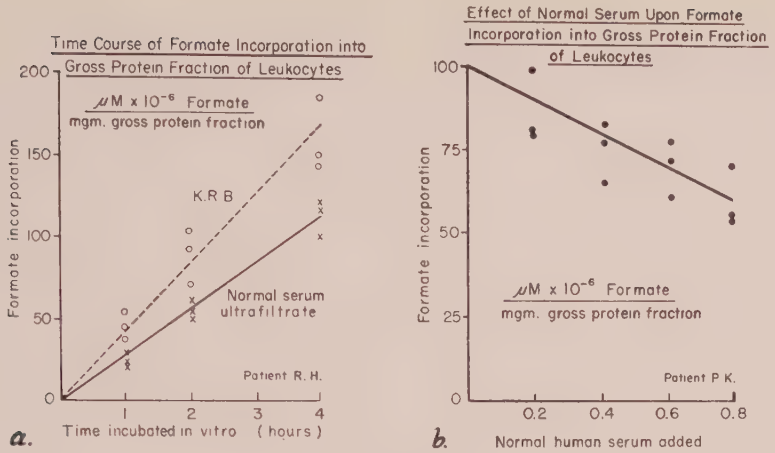


FIG. 1.

human sera on formate incorporation into different types of leukemic leukocytes. Preliminary data bearing on this question have been plotted in Fig. 2. These data include sera from normal subjects and from patients with various types of leukemia, lymphoma, lupus erythematosus, and other hematologic diseases. The categories tend to overlap considerably. However, 17 serial observations using one normal subject's sera on various cell samples representing the 3 major typs of leukemia have a mean inhibition of formate incorporation into leukocyte gross protein fraction (46.1%) which is significantly ($p < .05$) more pronounced than that of the 16 serum samples from patients with various types of leukemia (33.0%). Rather low levels of inhibition were obtained with serum from occasional cases of leukemia and lymphoma. Formate uptake appeared to be inhibited to a greater than normal extent in sera from 2 patients with lupus erythematosus. Such inhibition might, of course, be of a different type than the inhibitory activity noted in normal sera. Inhibition by sera from a number of other patients of various types was within normal limits.

*Discussion.* The present studies must be considered preliminary, but they strongly suggest the presence in normal serum of a heat stable, ether insoluble substance or substances

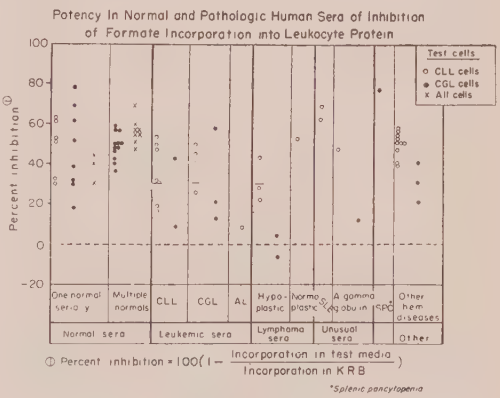


FIG. 2.

TABLE II. Effect of Normal Serum Ultrafiltrate on Precursor Incorporation into Gross Protein Fraction of Chronic Granulocytic Leukemia Leukocytes.

| Precursor compounds (.1 mg/beaker) | $\mu M \times 10^{-6}$ incorporation precursor/mg G.P.F. in K.R.B.G.* | % inhibition in serum ultrafiltrate† | Significance of difference from K.R.B.‡ |
|---|---|---|---|
| Formate-$C^{14}$ | 240 60 | 56 | $<.01$ |
| Glycine-1-$C^{14}$ | 4440 275 | 30 | $<.05$ |

* Mean ± stand. dev. of 3 replicates. † % inhibition $= 100\left(1 - \frac{\text{Incorp. in test media}}{\text{Incorp. in K.R.B.}}\right)$.

‡ Calculated from pooled intraprecursor stand. deviations.

TABLE III. Inhibition of Formate Incorporation into Leukemic Leukocytes by Normal Human Serum.

| | Cell type | | | | | |
|---|---|---|---|---|---|---|
| | CLL | | CGL | | AL | |
| No. of cell donors | 5 | | 4 | | 4 | |
| $\mu$M formate incorporated/mg protein* | 266 | 11 | 295 | 105 | 709 | 152 |
| % inhibition by normal serum | 43 | 24 | 38 | 26 | 48 | 18 |

* Mean $\pm$ stand. dev.

of relatively low molecular weight, which inhibit incorporation of radioactive formate and glycine into the gross protein fraction of all types of leukemic leukocytes to an appreciable and approximately equal extent. It appears that sera from some cases of leukemia demonstrate a decrease in this inhibitory effect. Whether such a decrease is secondary to the disease process or plays some part in pathogenesis is unknown.

A possible explanation for the inhibitory effect is the presence in serum of formate, glycine, or other nucleic acid precursors which effectively dilute out the added radioactive precursors. On the other hand, it is also possible that a metabolic inhibitor or regulator may be present. If the observed inhibitor activity were ultimately proven to represent a substance active in the normal regulation of leukocyte production or activity, one could speculate further regarding a possible role in pathogenesis of leukemia, as has been suggested by Osgood(2).

Further studies are being made.

*Summary.* The presence of one or more chemical substances in normal and pathological human sera capable of decreasing the rate of incorporation of formate and glycine into human leukemic leukocytes *in vitro* has been shown. Human leukemia sera as a group demonstrated a slightly subnormal inhibitor activity.

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## Preparation of a Functioning Frog's Heart Devoid of Ventricular Glycogen. (24219)

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In preparation for a study of factors relating to synthesis of glycogen by the ventricle of perfused frog's hearts, it was desirable to produce a functioning heart depleted of ventricular glycogen. The purpose of the present report is to describe the method used to produce such a heart and to call attention to a phenomenon which served to indicate when the ventricle was free of glycogen.

*Methods.* North American bullfrogs weighing 250-450 g were anesthetized by intraperitoneal injection of 20 mg sodium pentobarbital/100 g frog weight. The heart was exposed through a ventral midline incision, the circulation being left intact. After removal of the pericardium, the frog was placed in an airtight glass chamber where physiological salt solution(1) to which epinephrine was added in concentration of 1/500,000 dripped on the heart at a rate of 12-15 drops/min. The air in the chamber was then displaced by 100% $N_2$ and the chamber sealed tight. Though respirations ceased the heart continued to pump blood. After the desired time interval ventricular glycogen was determined by the method of Good, Kramer and Somogyi (2).

*Results.* After 0.5-4 hours in the chamber,

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TABLE I. Effect of Hypoxia on Cardiac Glycogen.

| Experiment (all frogs fasted 48-72 hr) | No. animals | Ventricular glycogen* mg % |
|---|---|---|
| 1. Controls | 6 | 1213 ± 104† |
| 2. After development of 3 × 3 mm white area and cannulation | 7 | 0 ± 0 |
| 3. Perfused with glucose, 100 mg % for 6 hr after depletion of glycogen | 6 | 588 ± 81 |

* All glycogen determinations were performed on ventricular tissue not involved by the white area.

† Stand. error calculated from the formula S.E. $\sqrt{\frac{\Sigma x^2}{N(N-1)}}$.

a white, opaque, non-pulsatile area which grossly resembled scar tissue developed at base of ventricle. If air was then admitted to the chamber the white area disappeared, only to reappear again when oxygen was displaced, after which it continued to enlarge until it involved the entire ventricle. At this point the heart ceased contracting and could not be revived by admitting oxygen. However, when the white area was still small, about 3 x 3 mm, the frog could be removed from the chamber, the beating heart cannulated in preparation for perfusion, and the ventricle demonstrated to be devoid of glycogen (Table I).

The ventricles contained approximately 1200 mg % glycogen prior to placement in the relatively anaerobic chamber. Upon development of the 3 x 3 mm white area and cannulation of the heart, all ventricles analyzed were depleted of glycogen (line 2). Line 3 indicates that hearts treated as in line 2 but then perfused aerobically for 6 hours with physiological solution containing 100 mg % glucose synthesized about 600 mg % of ventricular glycogen.

*Discussion.* It is well established that hypoxia decreases glycogen content of cardiac muscle. That epinephrine has a similar action was demonstrated by Bogue, Evans and Gregory in the dog heart-lung preparation (3) and by Chang in the rat(4). A combination of hypoxia and epinephrine was utilized in this study to produce the glycogen-free frog's ventricle. The epinephrine dripped onto the external surface of the heart and appeared to strengthen the contractions. In all probability the hypoxia played the greater role in depleting the ventricle of its glycogen.

Grossly the white area appeared to consist of contracted non-functioning muscle which did not transmit the color of the blood in the ventricle as did the remaining myocardium. The stronger and more rapid the contractions the sooner the white area developed. Occasionally a heart would beat many hours in the presence of lowered oxygen tension without developing this sign; yet analysis showed the ventricle to be free of glycogen. Thus the appearance of the white area indicated that the ventricles were devoid of glycogen, but its appearance did not necessarily coincide with the exact time of glycogen depletion. In general the hearts functioned well in the chamber except in a few instances where hemorrhage occurred during preparation of the specimen and significantly decreased the amount of blood flowing through the heart.

To be certain no gycogen was synthesized from possible precursors within the myocardium between the time the frog was removed from the chamber and the completion of cannulation the glycogen determinations were carried out after the latter procedure rather than before. The ventricles therefore contained no glycogen at the time perfusion with glycogenic substances was begun. Of interest is the fact that hearts treated as described not only beat but also were able to resynthesize glycogen from glucose.

*Summary.* A method utilizing epinephrine and hypoxia to produce a viable frog's heart devoid of ventricular glycogen in preparation for studies on glycogen synthesis is described. The development of a 3 x 3 mm white area at the base of the ventricle served to indicate when the myocardium was so depleted. Hearts treated by this method were able to resynthesize glycogen from glucose.

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# Effect of Dietary Bile Acids on *in vivo* Cholesterol Metabolism in the Rat.* (24220)

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Dietary cholesterol by itself does not cause any large amount of cholesterol deposition in rat liver, however, when fed with various bile acids, it produces cholesterol type fatty livers. Previously an increase in intestinal cholesterol absorption was accepted as the most obvious explanation of this phenomenon, but recent balance studies by Pihl(1) have given evidence that this explanation is untenable. Pihl suggested that a retarded rate of cholesterol catabolism coupled with normal rate of absorption would account for cholesterol accumulation in the presence of dietary bile acids. Since Friedman and Byers(2) have shown that accumulated cholesterol must be degraded as it is mobilized, Pihl's theory would seem acceptable. Further evidence is provided by other experiments(3,4) which have shown that bile acids prevent mobilization of accumulated liver cholesterol. The effect of cholic acid on metabolism of cholesterol has been studied by Byers *et al.*(5), who reported that cholic acid treated rats metabolize cholesterol at essentially the same rate as controls. However, their experiment was carried out on bile duct ligated animals which are not physiologically normal. In an effort to explain the hypercholesterolemic effect of bile acids, we have investigated the effects of cholic and dehydrocholic acids on synthesis and mobilization of cholesterol under normal physiological conditions.

*Methods.* In Exp. A, we studied the effect of cholic acid on cholesterol metabolism; while in a parallel Exp. B, we studied the effect of dehydrocholic acid. Female Sprague-Dawley albino rats—24, weighing 230-260 g in Exp. A, and 24, weighing 180-200 g in Exp. B, were divided into separate control and experimental groups for each of the 2 studies. Both control groups received a basal diet consisting of ground Rockland rat ration with 3% corn oil added; the experimental groups received the same diet supplemented with either 0.5% cholic acid (Exp. A), or 0.5% dehydrocholic acid (Exp. B). All animals were fed *ad lib.* for 3 weeks, during which average food consumption in each group was 18 g/day. Each rat then received an intraperitoneal injection of 50 $\mu$c of sodium acetate-1-$C^{14}$ in saline. Six, 24, 48 and 120 hours later, 3 control and 3 experimental rats for each of the 2 experiments were bled by heart puncture and decapitated. Samples of blood, liver, and adrenals were removed for analyses. Total serum cholesterol was determined by the method of Sperry and Webb (6). Serum bile acid was determined by absorption photometry by a combination of the methods of Minibeck and Wilken(7,8). Livers and adrenals were homogenized in a Lourdes multimixer, and total cholesterol determined on aliquots of homogenates by the method of Sperry and Webb. For determinations of radioactivity, blood, liver, and adrenal cholesterol was isolated as the digitonide and counted in a Tracerlab SC-16-Windowless Flow Counter. All samples were of such weight that no self-absorption corrections were necessary. After counting, the weight of cholesterol on the planchets was determined by the Lieberman-Burchard reaction. All radioactivity determinations are reported as specific activity of cholesterol (counts/min/mg cholesterol). To check the rate of acetate oxidation and size of the acetate pool, rate of incorporation of $C^{14}$ into expired $CO_2$ was determined for both experiments. In each experiment, 8 additional animals were fed the prescribed diets for 3 weeks. Then they received intraperitoneal injections of 50 $\mu$c of sodium acetate-1-$C^{14}$ in saline. The expired $CO_2$ was collected for 6 hours following injection. The collected $CO_2$ was precipitated as $BaCO_3$ and its activity determined in a windowless flow counter.

*Results. Exp. A.* As shown in Table I, 3 weeks of cholic acid feeding did not alter se-

* This investigation was supported in part by research grant from Mich. Heart Assn.

TABLE I. Effect of Dietary Cholic and Dehydrocholic Acids on Tissue Cholesterol Levels and on Serum Bile Acid Levels.

| Exp. | Group | Total cholesterol | | | Bile acid |
|---|---|---|---|---|---|
| | | Serum, mg % | Liver, mg/g | Adrenal, mg/g | Serum, mg % |
| A | Control | 81.6 ± 13.2 | 2.60 ± .19 | 44.7 ± 6.81 | 2.42 ± .61 |
| | Cholic acid treated (.5%) | 84.0 ± 16.9 | 2.83 ± .22 | 44.0 ± 5.95 | 4.25 ±1.03 |
| B | Control | 83.0 ± 10.2 | 2.97 ± .45 | 37.6 ± 6.27 | 2.42 ± .61 |
| | Dehydrocholic acid (.5%) | 84.6 ± 13.6 | 3.00 ± .11 | 42.7 ± 9.55 | 3.24 ± .89 |

Statistical comparison of values for control and treated animals:

Cholesterol: Exp. A: Serum, $P = .84$; Liver, $P = .015$; Adrenal, $P = .86$. Exp. B: Serum, $P = .75$; Liver, $P = .88$; Adrenal, $P = .15$.

Bile acid: Exp. A: Serum, $P < .01$. Exp. B: Serum, $P = .14$.

rum or adrenal total cholesterol levels, however, there was a slight but significant increase in liver total cholesterol. Cholic acid treatment resulted in a significant increase in serum bile acid level.

Table II shows that cholic acid greatly inhibited the rate of $C^{14}$ incorporation in serum, liver, and adrenal cholesterol. Furthermore, cholic acid greatly retarded rate of mobilization of cholesterol in these same tissues.

*Exp. B.* Dietary dehydrocholic acid did not increase serum, liver or adrenal cholesterol levels (Table I). In contrast to cholic acid, dehydrocholic acid produced no significant increase in serum bile acids. However, it must be kept in mind that dehydrocholic acid does not yield a product measurable by absorption photometry.

Dehydrocholic acid also reduced rates of synthesis and mobilization of cholesterol-x-$C^{14}$ in serum, liver, and adrenals, but to a lesser extent than cholic acid (Table II).

*Expired $CO_2$. Exp. A and B.* Acetate oxidation rate and acetate pool levels were compared in control and experimental groups by measurement of $C^{14}O_2$ activity. Table III shows that there was no significant difference between controls and treated animals in either experiment.

*Discussion.* From the reported results, it is clear that dietary cholic acid at the 0.5% level is a very potent inhibitor of both cholesterol synthesis and mobilization (*in vivo*). Also, at this level cholic acid does not cause accumulation of tissue cholesterol, when the diet is cholesterol free. This is due to the fact that the rates of synthesis and disappearance declined simultaneously, and thus

TABLE II. Serum, Liver and Adrenal Cholesterol-x-$C^{14}$ Levels in Normal and in Cholic and Dehydrocholic Acid Treated Rats as a Function of Time.

| Exp. | Group | Time, hr | Cholesterol-x-$C^{14}$, counts/min./mg cholesterol | | |
|---|---|---|---|---|---|
| | | | Serum | Liver | Adrenal |
| A | Control | 6 | 2,180 | 2,397 | 829 |
| | | 24 | 1,631 | 1,577 | 1,026 |
| | | 48 | 742 | 878 | 810 |
| | | 120 | 489 | 507 | 453 |
| | Cholic acid treated (.5%) | 6 | 459 | 803 | 129 |
| | | 24 | 500 | 571 | 301 |
| | | 48 | 325 | 483 | 234 |
| | | 120 | 311 | 357 | 295 |
| B | Control | 6 | 1,538 | 1,721 | 956 |
| | | 24 | 910 | 805 | 682 |
| | | 48 | 753 | 738 | 810 |
| | | 120 | 525 | 428 | 610 |
| | Dehydrocholic acid (.5%) | 6 | 953 | 1,081 | 505 |
| | | 24 | 732 | 729 | 458 |
| | | 48 | 570 | 566 | 536 |
| | | 120 | 427 | 408 | 485 |

TABLE III. Effect of Cholic and Dehydrocholic Acids on Rate of Oxidation of Sodium Acetate-1-$C^{14}$.

| Exp. | Group | Total $C^{14}O_2$, counts/min. | Significance |
|---|---|---|---|
| A | Control | $156.5 \times 10^5 \pm 8.8 \times 10^5$ | $P = .65$ |
| | Cholic acid treated (.5%) | $159.7 \times 10^5 \pm 9.8 \times 10^5$ | |
| B | Control | $129.4 \times 10^5 \pm 11.35 \times 10^5$ | $P = .10$ |
| | Dehydrocholic acid (.5%) | $146.0 \times 10^5 \pm 12.92 \times 10^5$ | |

maintained normal tissue cholesterol level. Whether or not the drop in synthetic rate is a primary effect of cholic acid can not be decided from these data. There was a very small but significant increase in liver cholesterol level, and it is well known that accumulated cholesterol will retard the rate of cholesterol synthesis.

The hypercholesterolemia resulting from simultaneous feeding of cholesterol and bile acids, can readily be explained by our results. The data in Table II show that cholic acid, and to a lesser extent dehydrocholic acid, reduced rate of mobilization of serum and liver cholesterol. Pihl has shown that dietary bile acids have little effect on rate of cholesterol absorption at the 0.5% level. Therefore, simultaneous feeding of cholesterol and bile acids must result in cholesterol accumulation.

In agreement with previous studies(1,2) cholic and dehydrocholic acids have similar but quantitatively different effects.

*Summary.* 1) A 3-week period of feeding a diet containing 0.5% cholic acid did not alter serum and adrenal total cholesterol levels. However, a slight but significant increase in liver total cholesterol was observed. Dietary dehydrocholic acid had no effect on serum, liver or adrenal total cholesterol levels. 2) Cholic acid increased serum bile acid concentration, while dehydrocholic acid had no effect. 3) Dietary cholic acid reduced rates of synthesis and mobilization of cholesterol in both liver and adrenal. Dehydrocholic acid produced similar results but to a lesser degree. 4) Accumulation and mobilization of serum cholesterol were retarded by dietary cholic acid, and slightly by dehydrocholic acid.

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## Effects of Gradual Complete Occlusion of Hepatic Veins in Dogs. (24221)

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The basic physiology of portal hypertension and ascites associated with portal cirrhosis is not satisfactorily explained. Proposed experimental methods for producing portal cirrhosis have failed to reproduce a clinical picture of portal hypertension(1-4). Madden *et al.*(5) working with corrosion specimens of the liver postulated that the primary factor in formation of ascites is an obstruction of the outflow tract of the liver, namely, the hepatic veins. This hypothesis appeared to us to be worthy of further experimental verification. After preliminary work on the anatomy of hepatic veins in dogs, we devised a relatively simple procedure for occlusion of these vessels.

*Materials and methods.* Adult mongrel dogs ranging in weight from 12.5 to 20.0 kg

were anaesthetized with sodium pentobarbital (30 mg/kg body weight) intravenously and the anterior abdominal walls were prepared by the usual sterile methods.

The abdomen was opened through a mid line incision. The portal vein and inferior vena cava were identified and venous pressures in the vessels taken by introduction of a thin walled 15-gauge needle into the lumen of the vessel through an area of vein wall enclosed by a purse string suture of 4-0 vascular silk. Prior to withdrawing the needle from the portal vein 20 cc of 70% Diodrast (Iodopyracet-Winthrop) was injected and a roentgenogram taken. The needle was then withdrawn and the puncture closed by the purse string suture.

A segment of the inferior vena cava cephalad to the renal veins was mobilized for a distance of 3 to 4 cm. Blalock vascular clamps were placed at upper and lower ends of this segment of vein. A piece of thin-walled, wide bore polythene cannula was then selected. A suture of No. 4-0 vascular silk was placed on the caudal end of the cannula to act subsequently as its anchor suture. Several small, silver, neurosurgical clips were placed on both ends of the cannula to facilitate radiological localization of the cannula when it was in place. The Blalock clamps were closed and an incision 1.5 cm long was made into the anterior wall of this isolated segment of the vena cava. The bevelled end of the cannula was introduced into the phlebotomy incision while an assistant released the cephalad Blalock clamp so that the cannula was fully inserted into the lumen of the vena cava (Fig. 1). The anchor suture of the cannula which had the needle still attached was brought out through the vein wall at the caudal end of the incision. The suture was then utilized to close the incision in the wall of the vena cava. A running stitch was employed for this purpose. In earlier cases we had not anchored the cannula in this fashion with the result that it was rapidly carried to the right atrium of the heart and led to the death of the animals. A neurosurgical type suction was found to be the best method for achieving visualization of the wound during closure of the opening in the vena cava. In each operation we selected the largest calibre of cannula which could be conveniently inserted into the vena cava. The abdomen was closed in layers without drainage.

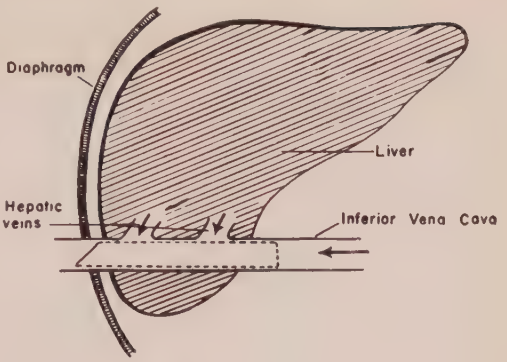


FIG. 1. Lateral projection of liver and inferior vena cava. Dotted lines illustrate position of plastic cannula. Wall of the cannula is in apposition to ostia of hepatic veins.

The following laboratory tests were done on each animal: (a) serum proteins with A/G ratio and electrophoretic fractionation; (b) serum sodium, potassium and chloride and (c) bromsulfalein dye retention(6). These studies were done preoperatively and at 2-week intervals following operation.

When ascites became clinically evident the animals were reoperated. Direct pressure readings were again taken from the inferior vena cava and portal vein. In a certain number of cases the portal venogram was repeated. When the animals were sacrificed necropsy specimens of liver, spleen, kidney and inferior vena cava in the region of the hepatic veins were taken.

Several dogs demonstrated at necropsy a complete obstruction of the vena cava associated with blockage of the hepatic veins. The possible role of the vena caval obstruction in production of the ascites and portal changes was, therefore, investigated and a control experiment was devised. Four dogs were selected and a short cannula of the same material was used. It was placed in the vena cava just cephalad to the renal veins, but well caudad to point of entry of the hepatic veins into the vena cava. The same biochemical studies were made as in the main experiment.

*Results.* Four animals survived the opera-

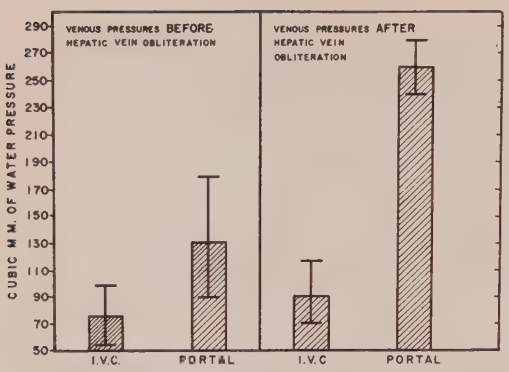


FIG. 2. Pressure (means and ranges for 10 dogs) in inferior vena cava and portal veins before and after obliteration of hepatic veins.

tion but died within a few days and before developing evidence of ascites.

Ten dogs developed clinically demonstrable ascites within 6 to 8 weeks of operation. In these the abdomen became markedly distended and tortuous venous collateral channels became prominent in the abdominal wall. When the ascites reached full clinical proportions, the animals developed a pitting oedema over the hind legs. Amount of ascitic fluid present varied with size of the animal, but ranged from 3500 to 5500 cc.

The pressures in the inferior vena cava and in the portal vein taken at the time of the original operation in this group averaged 75.3 mm and 130.5 mm of water respectively. When the animals were reoperated after establishment of ascites the pressures in these vessels averaged 90.5 mm and 260 mm of water respectively (Fig. 2). It was interesting to note that in this group the portal-systemic collateral venous channels were markedly dilated.

In each of the 10 animals the peritoneal cavity was filled with a clear yellow fluid of high specific gravity which had a protein pattern very similar to that of the plasma of the animal. The portion of the vena cava which contained the plastic cannula was thickened and examination of its intima showed that the ostia of the hepatic veins had been obliterated by organized thrombi in the lumen of each vein (Fig. 3). Various stages of thrombus formation and organization could be recognized in serial sections of the hepatic veins (Fig. 4). Sections of the liver taken at necropsy revealed marked congestion in the central region of the liver lobules. Fibrosis of the liver substance was not a prominent fea-

FIG. 3. Inferior vena cava has been opened in region of hepatic vein ostia. Scarred area at tip of pointer marks position of an obliterated hepatic vein ostium.

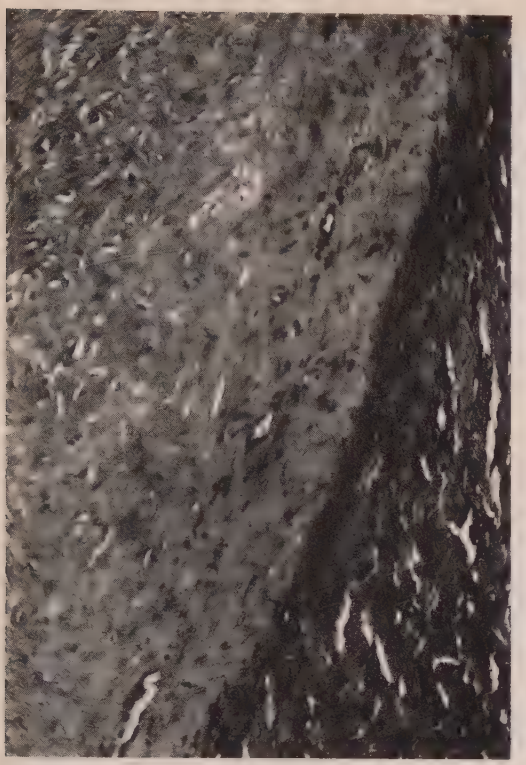

FIG. 4. Thrombus organization in lumen of an hepatic vein. The darker tissue represents the original wall of hepatic vein, the lighter tissue the organized thrombus.

ture in any of our animals. Sections of the spleen and kidneys revealed moderate congestion.

Four control dogs remained healthy for a 6-month period following operation. There was no evidence of clinical ascites or portal hypertension and their biochemical tests remained normal. When sacrificed the cannula was patent in 2 of the animals and completely thrombosed in the other 2, indicating that in these 2 the factor of vena caval obstruction had been reproduced. In none of these animals was there evidence of occlusion of the hepatic veins. This would indicate that the pathology resulting in the original series was due to occlusion of the hepatic veins and not to occlusion of the inferior vena cava.

The animals showed an overall decrease in total serum protein with a reversal of the albumin-globulin ratio and an abnormal electrophoretic pattern: significant decrease of albumin and rise of alpha$_2$ and gamma globulins. The bromsulfalein dye retention(6) increased from 10% to 70% as the ascites progressed. Serum sodium, potassium and chloride showed little fluctuation and remained essentially normal throughout the procedure.

*Discussion.* Presented data show that marked ascites and a degree of portal hypertension are produced in all animals in which this procedure is correctly employed. It is necessary to stress the importance of accurately placing the cannula so that fibrosis of the ostia of all the hepatic veins is produced. Failure to obliterate even one large hepatic vein may permit enough venous outflow from the liver to prevent formation of ascites and portal hypertension. We feel the control experiment described demonstrates that the important factor in production of portal hypertension and ascites is the occlusion of the hepatic outflow tract and that the inferior vena caval obstruction which occurs in some of the animals is not an important factor. This procedure is relatively simple technically and produces uniform results. It provides a source of clinical and experimental material by which we may assess the relative merits of the types of therapy currently prescribed for these conditions.

*Summary.* A new method for producing gradual, complete occlusion of the hepatic veins is described. Associated with this is the successful production of ascites and a degree of portal hypertension.

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# Superiority of Fluorescein Isothiocyanate (Riggs) for Fluorescent-Antibody Technic with a Modification of its Application. (24222)

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The fluorescent antibody technic of Coons and Kaplan(1) has been widely used for identification of microbiological agents(2-7). Until recently, fluorescein isocyanate was the only compound that could readily be conjugated to the antibody molecule and yield a product that was easily detectable with ultraviolet microscopy. The difficulties involved in preparation of fluorescein isocyanate and its subsequent conjugation have somewhat restricted the application of this technic. A further limiting factor has been the need for antisera of relatively high titer, since during conjugation a portion of the antibody globulin is denatured to such an extent that it is no longer reactive. To eliminate these disadvantages Goldman and Carver(8) modified the method of conjugation by use of a filter paper technic. Riggs(9) described an isothiocyanate derivative of fluorescein with a number of qualities that make it superior to fluorescein isocyanate. The purpose of this study was to determine the relative merits of each of the compounds employed in various methods of conjugation, and to seek possible improvements in the technic.

*Material and methods.* The following antisera were used throughout the study: *Pasteurella tularensis* and Venezuelan equine encephalomyelitis (VEE) received from the Army Chemical Center, Fort Detrick, Salmonella polyvalent and sheep antihuman furnished by Walter Reed Army Institute of Research, antirabbit from Cappel,* and *Cryptococcus neoformans* and *Saccharomyces cerevisiae* prepared in this laboratory. *Preparation of fluorescein compounds*: A single lot of fluorescein amine isomer No. 2, prepared by one of us (CWS), was used to make both the fluorescent isocyanate and the fluorescein isothiocyanate. The fluorescein isocyanate was synthesized by the method of Coons and Kaplan(1). A fluorescein isothiocyanate derivative was prepared by the method of Riggs (9): 2 ml thiophosgene was added to 5 ml dry acetone and placed on a rotary shaker. One gram of fluorescein amine was suspended in 5 ml dry acetone and the suspension was added drop by drop to the thiophosgene-acetone mixture. A dark yellow precipitate formed. The reaction was permitted to continue at room temperature with constant shaking for one hour. Then the mixture was placed in refrigeration at 4°C and allowed to stand overnight. The precipitate was collected under suction, washed with 4 to 5 volumes of Skellysolve B (petroleum naphtha)† and dried over calcium chloride. The yield was approximately 96%. The product was stored in a screw-capped vial at room temperature. *Conjugation of globulin fractions*: The ammonium sulphate precipitated globulin fraction of each antiserum was divided into 6 aliquots. Fluorescein isocyanate was used to label one of them by the method of Coons and Kaplan(1). A second aliquot was labeled with fluorescein isocyanate by the filter paper technic of Goldman and Carver(8). Fluorescein isothiocyanate was conjugated to a third aliquot by the procedure described by Riggs(9). A fourth portion of the globulin fraction was conjugated with fluorescein isothiocyanate, using the filter paper technic. A fifth sample was conjugated in the following manner: The globulin fraction was diluted with 0.15 M sodium chloride and carbonate-bicarbonate buffer, 0.5 M, pH 9, so that the final solution contained 10 mg protein per ml and 10% buffer solution. After the solution was cooled to 4°C, 0.05 mg fluorescein isothiocyanate was added for each milligram of protein. The mixture was transferred to a rotary shaker, placed in the 4°C cold room, and agitated for 12 to 18 hours to ensure uniform distribution of the dye. The conjugated globulin fraction was then transferred to a

* Cappel Laboratories, Inc., Westchester, Pa.

† Skelly Oil Co., Eldorado, Kan.

TABLE I. Comparison of Fluorescent Staining Titers of 7 Sera Conjugated by 5 Different Methods.

| | Fluorescein isocyanate | Fluorescein isocyanate filter paper | Fluorescein isothiocyanate | Fluorescein isothiocyanate filter paper | Fluorescein isothiocyanate powder |
|---|---|---|---|---|---|
| *P. tularensis* | 5 | 20 | 80 | 160 | 160 |
| Salmonella polyvalent | 10 | 20 | 40 | 80 | 80 |
| *Sacc. cerevisiae* | 40 | 40 | 160 | 80 | 160 |
| *C. neoformans* | * | 10 | 20 | 20 | 40 |
| V. E. E. | * | * | * | 5 | 5 |
| Antihuman | 10 | 20 | 80 | 80 | 80 |
| Antirabbit | 40 | 80 | 160 | 160 | 160 |

Titers expressed as reciprocal of serum dilution. * Undiluted.

cellulose casing and dialyzed against 0.01 M phosphate-buffered saline solution, pH 7.0, until the dialysate was free of fluorescence when exposed to ultraviolet light. The final portion of globulin fraction was retained unconjugated for studying other types of antibody activity. *Fluorescent microscope*: A Leitz Ortholux microscope, equipped with a reflecting darkfield condenser, 4 mm dry objectives, 10 x widefield ocular and appropriate filters was used in conjunction with a 150-watt high pressure mercury vapor bulb mounted in a Leitz fluorescent lamp assembly.

*Results*. Smears were prepared from various antigens and fixed in absolute methanol for 10 minutes. After the smears were thoroughly air dried, they were covered with different dilutions of the labeled homologous antiglobulin and kept in a wet chamber at room temperature for 30 minutes. They were washed for 10 minutes in 0.01 M phosphate buffered saline, then mounted in 10% buffered glycerin. The smears thus prepared were examined under the ultraviolet microscope. The results (Table I), demonstrate that fluorescein isothiocyanate conjugated antisera yielded positive results in higher dilutions than the fluorescein isocyanate conjugates.

In the case of *P. tularensis*, the slides stained with undiluted antisera conjugated with fluorescein isothiocyanate fluoresced so brightly that a distorting halo was observed. This effect disappeared upon using higher dilutions. Conjugated antisera diluted to as much as 1:160 still stained the organisms so that the structure of the individual cells was easily recognized.

Similar results could not be obtained with the fluorescein isocyanate preparations when diluted more than 1:20. The reactions were specific, for no staining was observed when organisms other than the homologous species were tested.

The antihuman and antirabbit globulin preparations were tested by the indirect technic. Smears of *Salmonella typhi* were exposed for 30 minutes to the serum of an individual who had recently been immunized. The slides were washed for 30 minutes in phosphate-buffered saline to remove excess antiserum. The organisms coated with a layer of human antibody were then used as a homologous antigen. The conjugated antihuman sera were tested by the standard method. The antirabbit serum was tested in a similar manner using *Paracolobactrum aerogenoides* and the homologous rabbit antiserum.

Agglutination studies were performed with all antisera except the VEE and the antirabbit globulin. Those antisera that had been exposed to organic solvents during conjugation did not retain their complete titer when compared with the unconjugated fraction, while those conjugated by using either the filter paper technic or with the fluorescein isothiocyanate powder retained approximately the same titer as the control fraction.

Results are shown in Table II.

*Discussion*. Various methods for conjugation of fluorescein dyes have been tested. Fluorescein isothiocyanate has certain attributes which make it superior to fluorescein isocyanate for conjugation with immune sera. A more intense staining with fluorescein isothiocyanate conjugated antisera was demon-

TABLE II. Effect of Conjugation on Agglutination Titers of 5 Sera.

| | Fluorescein isocyanate | Fluorescein isocyanate filter paper | Fluorescein isothio-cyanate | Fluorescein isothio-cyanate filter paper | Fluorescein isothiocya-nate powder | Uncon-jugated |
|---|---|---|---|---|---|---|
| *P. tularensis* | 160 | 320 | 160 | 640 | 640 | 640 |
| Salmonella polyvalent | 2 | 20 | 10 | 20 | 20 | 20 |
| *Sacc. cerevisiae* | 320 | 640 | 640 | 640 | 640 | 1280 |
| *C. neoformans* | 40 | 160 | 80 | 160 | 160 | 160 |
| Antihuman | 4 | 256 | 2 | 256 | 128 | 256 |

Titers expressed as reciprocal of serum dilution.

strated by the fact that they could be employed in much higher dilution. In some instances, the undiluted preparations were unsatisfactory because the fluorescence was so intense that distortion in the form of a halo was observed.

Our comparative study of the stability of various compounds confirmed Goldman's report(8) on the filter paper technic for storage of fluorescein isocyanate. The fluorescein isothiocyanate, on the other hand, has remained stable as a powder for a period in excess of 8 months. This obviates the necessity of applying the dye to filter paper for storage. One of the limiting factors in the use of the fluorescent antibody staining technic has been the necessity of maintaining highly purified organic reagents for use in conjugation. This requirement has been eliminated. Fluorescein isothiocyanate powder poured directly into a cooled, dilute, buffered antiserum yielded a product which was as good as, or superior to that prepared by any other method.

The ease with which serum may be conjugated by this method enables any laboratory to prepare and use it with a minimum of supplies, equipment, and personnel. The stability of the fluorescein isothiocyanate compound lends itself to centralized preparation. This compound and method will permit labeling of low titer antisera because little detectable denaturation of the protein occurs.

*Summary.* 1) The globulin fractions of antisera representing bacterial, viral, mycotic agents and antiglobulin fractions were labeled with 2 derivatives of fluorescein amine by 3 methods. 2) Fluorescein isothiocyanate was shown to be superior in stability, ease of conjugation, and degree of fluorescence. This direct method of adding the dye to a dilute buffered antiserum eliminates the need for organic reagents which may denature the protein.

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# Effects of Tolbutamide on Intestinal Glucose Absorption and Blood Glucose Levels.* (24223)

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Apparent inhibition of intestinal glucose absorption by oral or intravenous carbutamide (1) has been attributed to possible effects of this compound on gastrointestinal motility (2) since some of its congener sulfonamides have been shown to affect visceral smooth muscle motility(3). The work with carbutamide suggests particularly a delay in passage of fluid from the stomach(1,2). Experience with still another member of this group, tolbutamide, lends support to this view. Thus, in the present study, with glucose absorption confined to the small intestine of the rat, tolbutamide introduced directly with the glucose solution into the gut lumen had no significant effect upon absorption of the sugar in acute experiments. At the same time, the amount of tolbutamide absorbed was sufficient to affect blood sugar levels measured at the end of the absorption period.

*Methods.* Adult, male rats‡ weighing from 170 to 278 g were used. The experiments were done on paired rats with all procedures including analyses being done at the same time for each pair. In each of the 9 pairs used, one rat served as "control" and the other as "test." The pairing was done by weight so that no more than 8 g difference existed and this difference was randomly distributed so as to favor neither "controls" nor "tests." The rats fasted 24 hours before each experiment, and were anesthetized with sodium pentobarbital (30 mg/kg, I.P.). The small intestine was reached through a midline incision in the ventral abdominal wall. The common bile duct was ligated and 2 glass cannulae were used to isolate the entire small intestine and connect its lumen with 10 ml syringes at both ends. The loop thus made was rinsed with warmed normal saline. When these washings were clear, a final rinse was made with a 1% glucose solution. This was flushed out with air in the same manner as the fluid in the system would be flushed out at the end of the absorption period in each experiment. Immediately following these preliminary steps, the solutions for absorption were introduced. One of the loop systems ("control") was filled with 1% glucose solution and the other ("test") received a solution of 1% glucose and 0.1% tolbutamide.§ The distal syringe barrel, without plunger, served as a means of establishing and maintaining a uniform hydrostatic pressure of 7 cm of the solution during the absorption period. Solution was added as necessary from the proximal syringe. After 45 minutes each system was emptied as described above. Both the solutions introduced and those removed, as well as tail vein blood samples taken before and at the end of the absorption period, were analyzed for glucose by the Somogyi-Nelson method(4). Net glucose absorption was estimated as the difference between the quantity introduced and that recovered from each loop system.

*Results.* Average net glucose absorption values are shown in Table I. The difference between the means is not significant ($t = 0.3$; $p > 0.7$) by the method of group comparison analysis(5). The mean difference between initial and final tail vein blood glucose concen-

TABLE I. Net Intestinal Glucose Absorption without ("Control") and with ("Test") Tolbutamide.

| Group | n | Net glucose absorbed, mg |
|---|---|---|
| Control | 9 | 152 ± 16* |
| Test | 9 | 150 ± 14 |

* Mean ± stand. error.

* Supported in part by a grant from the U. S. Public Health Service.

† Public Health Fellow. Present address: Fairfield Univ., Fairfield, Conn.

‡ Hooded rats from the colony maintained by the Dept. of Psychology.

§ Tolbutamide (Orinase-Upjohn) was kindly supplied by the Upjohn Co.

TABLE II. Mean Difference between Initial and Final Blood Glucose Concentration in Control and Test Rats.

| Group | n* | Mean difference, mg % |
|---|---|---|
| Control | 8 | 85 ± 14.2† |
| Test | 8 | 51 ± 4.8 |

* Blood glucose analyses were not obtained in one exp. pair included in Table I.
† Mean ± stand. error.

trations is shown in Table II. The increase is significantly greater ($t = 2.24$; $p<0.05$) by group comparison analysis(5) in the control group. For all the rats in which blood glucose concentrations were obtained, mean initial value was 94 mg %. Although not quantitatively measured, the peristaltic activity as reflected by oscillations in the fluid level in the distal syringe barrel appeared to be about the same in both control and test systems.

*Discussion.* It is apparent that under the test conditions in the rat, both tolbutamide and glucose are readily absorbed from the same solution. The net glucose absorption data support this directly in the case of glucose. The significantly smaller increment of blood glucose rise over the period of equivalent net glucose absorption is indirect evidence that tolbutamide was absorbed in sufficient quantity to exert its typical effect on blood glucose in normal rats. The apparent inhibition in absorption of glucose in the experiments with carbutamide previously cited(1) may be due to prolongation of gastric emptying time but this awaits direct proof. Structural differences in this family of compounds are too well known to be associated with widely varying physiological action to allow conclusions to be drawn by analogy from tolbutamide to carbutamide.

*Summary.* In rats glucose absorption from the small intestine was not affected by the presence of tolbutamide in the glucose solution used. The tolbutamide absorbed in these experiments was sufficient to produce a relative suppression of the alimentary hyperglycemia present at the end of the absorption period.

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## Survival of Unfertilized Mouse Eggs During Freezing and Thawing.* (24224)

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Preservation by freezing has been possible only with certain cells, others being susceptible to lethal factors, usually associated with ice formation. Long-term storage of germ cells at sub-freezing temperatures was given impetus by introduction of glycerol as a protective agent(1). Today, many of our dairy cattle are bred artificially with semen so preserved(2), and the technic has had successful clinical application in insemination of humans(3).

* This study was supported in part by a research grant from the Nat. Inst. of Health, P.H.S.

Similar success has not been realized with the mammalian female gamete, although reports on fertilized rabbit eggs and eggs of lower forms are encouraging. Eggs of the sea urchin reportedly were fertilized upon rewarming from a medium of sea water plus glycerol which had been frozen at –10°C(4). Formation of ice in the jelly coats of fertilized eggs of the frog did not prevent their subsequent normal development into tadpoles(5). Some hydrated eggs or early embryos of the brine shrimp proceed to hatch into free-swimming larvae after thawing from their frozen

salt water environment at –196°C(6). Smith (7) reported survival, on the basis of cleavage *in vitro*, of some (20-30%) fertilized rabbit eggs exposed to –20°C in frozen glycerolated medium. Also, upon rewarming from –190°C, 1% cleaved. However, it is not known whether fertilized rabbit eggs so treated are capable of developing into progeny when transferred into a foster mother.

We recently reported that unfertilized eggs of the mouse can be cooled to –10°C in an ice-free medium without alteration of their functional capacity(8). The purpose of this communication is to report their capability to be fertilized and to develop into progeny after the medium is frozen at this temperature.

*Materials and methods.* The technic of transplanting genetically labeled eggs from immature brown C57 females to mated, fertile, white BALB/c foster mother recipients, was employed in evaluating survival(9). Recipients were autopsied on the 19th day of gestation for detection of transplanted eggs, which developed as dark-eyed fetuses. Per cent survival was computed by dividing the number of transplanted eggs which developed, by the total number of eggs transplanted in pregnant recipients, and multiplying quotient by 100. Modified Locke's solution containing 5% glycerol by volume, was used as a medium(8). Ten to 12 eggs were placed in 1.5 ml glass ampuls containing 0.3 ml of the medium. Sealed by flame and clipped to fiberglass canes, the ampuls were passed through a bath at 5°C and transferred to a –10°C alcohol bath for 15 minutes. The ampuls were then lifted above the bath surface and the supercooled content frozen by seeding with a stick of solid carbon dioxide by contact with the side of the ampul. The frozen mass (Fig. 1A) was then reimmersed in the –10°C bath, before thawing in a 5°C water bath. Thermograph recording through a thermocouple in a test ampul with eggs and 0.3 ml medium, showed that average rate of temperature changes was 8°C/min from 21 to 5°C, 2°C/min from 5 to –10°C and 5°C/min from –10 to 0°C. After thawing, the eggs were removed from the ampuls with a small amount of medium and 6 from each transferred into recipients in tests of progeny development(9).

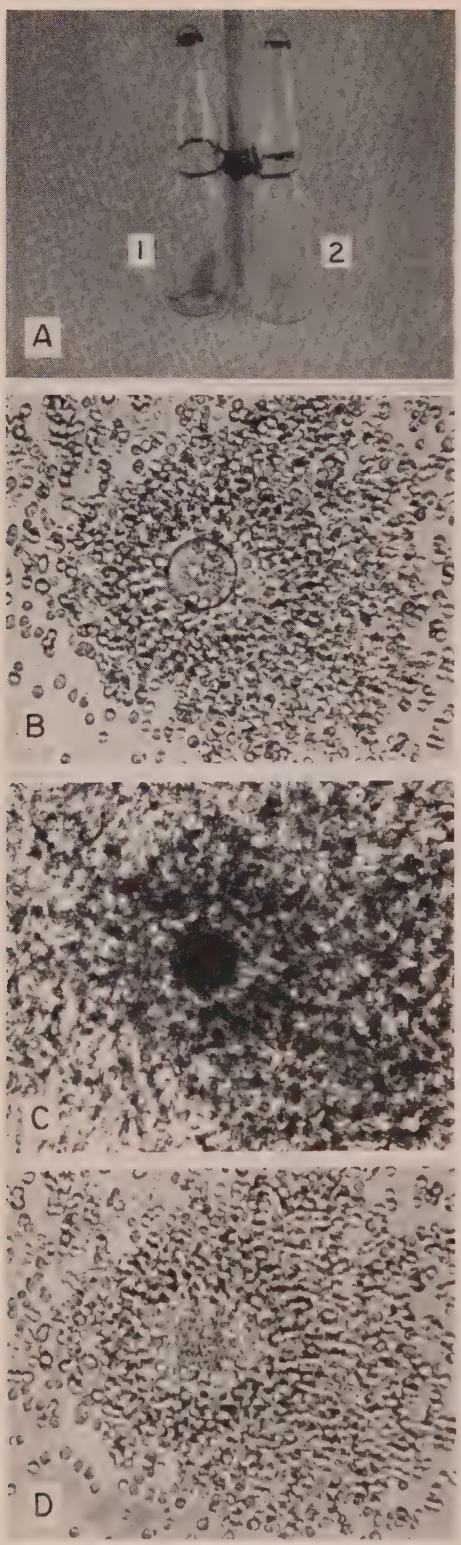
TABLE I. Survival of Unfertilized Mouse Eggs in Frozen Medium at –10°C.

| Exposure time (hr) | No. of recipients | | No. of eggs transplanted in pregnant recipients | | |
|---|---|---|---|---|---|
| | Total | Pregnant | Total | Developed | % survival |
| ½ | 7 | 6 | 36 | 7 | 19 |
| 3½ | 14 | 10 | 60 | 7 | 12 |

Freezing in the ampul did not favor accumulation of more detailed information on the relationship between site of ice formation and egg survival. To accomplish this, a special cooling chamber† was used which permits microscopic observation during freezing and thawing. Cover slip preparations of eggs with medium were supercooled to and seeded at –10°C. Because of differences in conditions in the ampul and between 2 cover slips (volume of fluid, rates of cooling and rewarming, velocity of ice formation, etc.), observations were also made on several types and sizes of hanging and inverted hanging drop preparations which more closely resembled conditions in the ampul. Since, under imposed conditions, no internal ice formations were seen as distinct structures with ordinary and polarized light, intracellular crystallization was considered as characterized by "blacking out" of these cells. Ice formation produces this opacity(10).

*Results.* Eggs in ampuls thawed from medium frozen at –10°C were indistinguishable from controls. Survival of eggs, as tested by transplantation, after exposure to frozen medium at –10°C in ampuls for ½ and 3½ hours (Table I) agrees well with that range of values (11-17%) found for several control series. Without exception, eggs thawed from medium seeded at –10°C in cover slip preparations were damaged, their contents being disorganized and vitelline membrane unrecognizable (Fig. 1D). These eggs were observed, also without exception, to freeze internally (Fig. 1C) after the supercooled condition (Fig. 1B) was seeded. Observations on the

† This chamber was designed and constructed by Mr. Maxim Persidsky in the biophysics laboratory at this foundation.




hanging and inverted hanging drop preparations of eggs during freezing to and thawing from −10°C, reveal the following: a) With occasional exception, eggs frozen internally suffer severe damage, b) Frequently, intracellular ice forms in some eggs but not in others of the same preparation, c) At times, it appears that intracellular ice forms in the surrounding cumulus cells but not in the eggs, and d) Very often, there is a delay of one to as many as 15 seconds between the internal freezing of different eggs which freeze in the same preparation.

*Discussion.* To our knowledge, intracellular ice has never been demonstrated in mammalian spermatozoa or any other cells during procedures employed in their successful preservation by freezing. Our results, although not ruling out the remote possibility suggested by occasional maintenance of structure following internal crystallization, indicate that unfertilized mouse eggs, treated as described, do not survive detectable intracellular ice formation. Probably, eggs that survived exposure to frozen medium in the ampul had not frozen but remained in a supercooled condition. Our recent work (unpublished observations) reinforces the notion that most, or all, unfertilized mouse eggs are physically injured by detectable internal crystallization. Over 90% of eggs cooled slowly to −20°C at a rate of 0.7°C/min remain unaltered structurally while those cooled rapidly at 12°C/min to the same temperature are almost always destroyed. The proposed explanation is that, in association with little or no penetration of glycerol(9), such slow cooling permits further cellular dehydration during the extracellular hypertonicity accompanying extracellular ice formation, obviating or minimizing the subsequent internal ice formation, while rapid cooling prevents this dehydration, hence favoring appreciable internal crystallization with damage to the cytoplasm.

We may now add unfertilized mouse eggs to

FIG. 1. (A) Ampuls with unfertilized mouse eggs in 0.3 ml modified Locke's + 5% glycerol, at −10° C, (1) unfrozen, (2) frozen; (B) egg in unfrozen medium at −10°C in cover slip preparation within cooling chamber; (C) same egg at −10°C after freezing; (D) same egg after thawing.

the growing list of mammalian cells which survive "freezing" in presence of glycerol. However, "freezing" may only mean survival in presence of extracellular ice without intracellular ice formation. Evidence for egg survival after intracellular ice formation would have to be based on observation of ice formation under the microscope, followed by transplantation of the same individual intact eggs and their normal reproductive performance.

*Summary.* This investigation has established the possibility of survival of unfertilized mouse eggs with extracellular ice formation in glycerolated modified Locke's solution at –10°C for periods up to 3½ hours. Survival of eggs so treated was evaluated by progeny development after transplantation into foster mothers.

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## Plasma Cells and Serum Proteins in Marine Fish.* (24225)

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There is evidence that in human beings and other mammals plasma cells may participate in the production of gamma globulins and antibodies(1,2). Studies on plasma cells and gamma globulins in lower forms were initiated(3-5) to determine whether or not they are associated. This report describes observations on occurrence of plasma cells and on electrophoretic properties of blood serum proteins in some elasmobranchs and teleosts.

*Materials and methods.* From one to 10 members of each species were examined shortly after capture. The fish were trapped in the region of Woods Hole, Mass., the majority from Buzzard's Bay. Blood was obtained from living animals by cardiac puncture with silicone-treated equipment. Collection of blood was greatly facilitated by anesthetizing the fish in a tank of sea water containing 1 part per thousand of M.S. 222§ before obtaining the sample. The blood was transferred to plastic tiubes, was allowed to clot, was centrifuged, and the serum withdrawn. It was either analyzed on the day of withdrawal or stored at –10°C for a few days. One specimen, serum of the lemon shark, *Negaprion brevirostris,* was obtained from the Serological Museum, Rutgers University.‖ This sample had been sterilized by filtration and stored at 4°C for several months. The serum proteins were analyzed by zone electrophoresis in starch gel. Soluble starch was produced by acetone-HCl treatment of po-

* This investigation was supported in part by grant from the Nat. Cancer Inst., P.H.S.

† Lalor Fellow, 1957.

‡ Serological Museum, Rutgers Univ., New Brunswick, N. J. Aided by grant from National Science Fn.

§ Meta-aminobenzoic acid-ethylester methane sulfonate obtained from Sandoz Pharmaceutical Co., Hanover, N. J.

‖ We are indebted to Dr. Alan Boyden, for making this specimen available.

TABLE I. Cell Size Summary. Size of cells as measured on smears and touch preparations of blood and spleen.

| | Red blood cells | Lymphocytes | Eosinophils | Neutrophils | Plasma cells |
|---|---|---|---|---|---|
| Human | 8.3 $\mu$ | 9.9 $\mu$ | | 13.0 $\mu$ | 14.6 $\mu$ |
| Smooth dogfish | 12.5 × 18.2 | 10.9 | 15.6 $\mu$ | | 14.6 |
| Dusky shark | 12.0 × 17.7 | 11.5 | 12.5 | | 14.6 |
| Sting ray | 12.5 × 16.2 | 9.4 | 12.0 | | 14.1 |
| Goosefish | 9.4 × 12.5 | 6.3 | | 9.9 | 6.3 |
| Bonita | 8.3 | 7.3 | 11.5 | | 9.4 |
| Flounder | 7.3 | 6.8 | | 9.9 (?) | 9.0 |
| Tautog | 7.3 | 6.3 | | 10.4 | 8.3 |

tato starch¶ at 36.1°C for 70 minutes. Gels were prepared from this soluble starch in 0.030 M sodium borate buffer of pH 9.05, and electrophoresis was carried out at 10°C for 16 hours with a potential of 4.5 volts/cm(6,7). Blood smears were obtained, and touch preparations of the fresh spleens were made by lightly touching a newly cut surface to glass slides. The touch preparations and blood smears were dried, treated with Wright-Giemsa stain, and studied at a magnification of 1000x. Occasionally touch preparations of the liver were also studied. Criteria for identification of plasma cells were the same as for their recognition in mammalian lymph nodes, bone marrows, and spleens. They were identified by the occurrence of: (a) an eccentric nucleus showing varying degrees of clumping of the chromatin; (b) abundant basophilic cytoplasm, having a somewhat granulated appearance without the presence of true granules; and (c) usually a paranuclear clear zone. The frequency of occurrence of plasma cells was estimated, using a scale of 0 to 4 plus. Among the species sampled, *Mustelus canis* demonstrated the greatest frequency of plasma cells in the spleen and thus was assigned the 4 plus designation. Relative sizes of white blood cells in smears and imprints of some specimens were determined using a micrometer.

*Results.* All of the white blood cells in the spleens of the elasmobranchs were about the same size as their counterparts in human tissues (Table I). Structures having the characteristic appearances of plasma cells were readily identified (Fig. 1-C,D,E).

Among the teleosts, however, the white blood cells of the spleens were considerably smaller than those of human beings (Table I), and identification of plasma cells was less certain. Nevertheless, a few small cells from spleens of each of the teleosts appeared to possess some of the morphologic features of plasma cells (Fig. 1-F,G,H,I,J): the nuclei were eccentric; the cytoplasms were basophilic and usually not granulated in appearance; and the paranuclear clear zones were less prominent than in typical mammalian plasma cells. It was possible to identify plasma cells in the spleen of *Sarda sarda* (bonita) with greater certainty than in the other species of teleosts studied (Fig. 1-J).

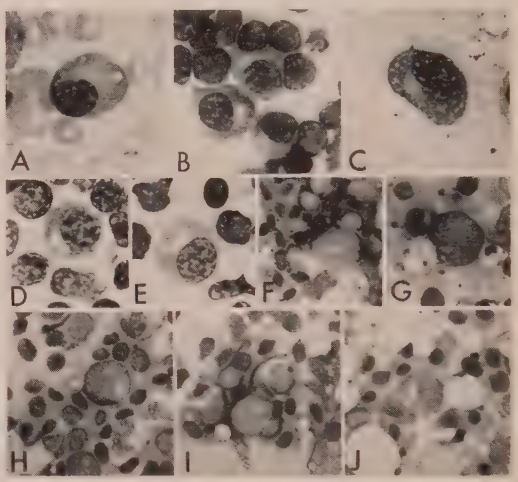


FIG. 1. Plasma cells in smears and touch preparations stained with Wright-Geimsa, 500 ×.

A. Bone marrow, human. B. Lymph node, rabbit. C. Liver, smooth dogfish. D. Spleen, smooth dogfish. E. Spleen, dusky shark. F. Spleen, ramoura. G. Spleen, goosefish. H. Spleen, tautog. I. Spleen, flounder. J. Spleen, bonita.

The serum from each species of elasmobranch and teleost had its own characteristic zone electrophoretic pattern, but within a

¶ Lot No. 584 potato starch obtained from Morningstar-Nicol.

| | Plasma cells (spleen) |
|---|---|
| *Mustelus canis,* smooth dogfish | ++++ |
| *Squalus acanthias,* spiny dogfish | ++ |
| *Carcharhinus obscurus,* dusky shark | +++ |
| *Raja oscellata,* skate | +++ |
| *Torpedo nobiliana,* torpedo ray | +++ |
| *Dasyatis centroura,* sting ray | |
| *Raja clavata,* skate | |
| *Negaprion brevirostris,* lemon shark | |
| *Homo sapiens,* man | |

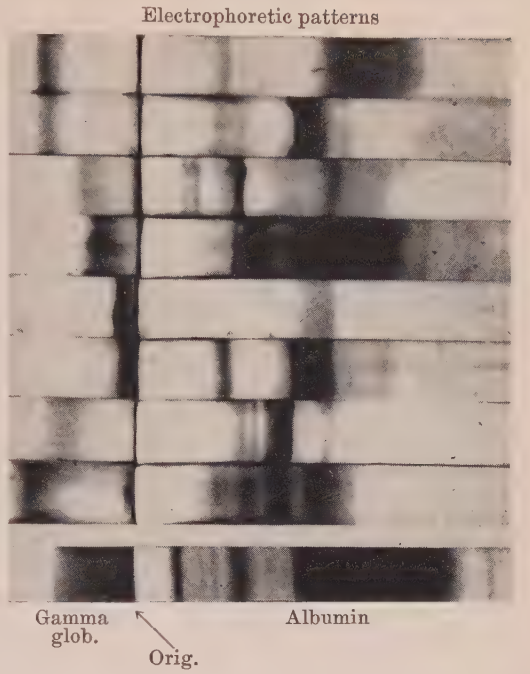


FIG. 2. Starch gel electrophoretic patterns of sera from some elasmobranchs. The position at which samples were applied is indicated by arrow below figure. Cathodic area is left of the origin and anodic area is toward the right.

species there was only slight variation. Zone electrophoresis of serum from each of the elasmobranchs resulted in migration of proteins into the gamma globulin region (cathodic region) of the starch gel. These proteins, migrating toward the cathode, became isolated into well-defined, narrow zones (Fig. 2) in contrast to the diffuse, continuous zone characteristic of human gamma globulin.

Among the teleosts, proteins corresponding electrophoretically to human gamma globulins were either greatly reduced or absent (Fig. 3) Only in the case of *Sarda sarda* (bonita) were there detectable bands in the gamma globulin area, and these were very faint. The occurrence of a protein with a mobility corresponding approximately to that of human serum albumin, and of another which was slightly slower than human B globulin, was consistently observed in every species of bony fish studied.

Figs. 2 and 3 summarize the results of our observations on the occurrence of plasma cells in the spleens and the presence of gamma globulins in the samples of serum from each species. Among the elasmobranchs and teleosts, whenever the frequency of occurrence of plasma cells in the spleens was 3 plus or 4 plus proteins corresponding to mammalian gamma globulin were found in the serum. Whenever the frequency of plasma cells in the spleens was 0 to 2 plus, such proteins could not be detected in the serum.

*Discussion.* Utilizing boundary electrophoretic studies Deutsch and McShan(8) noted the absence of gamma globulin in the sera of fish. They identified up to 12 components in the electrophoretic patterns of a single species. Variation from one individual to another within the same species was slight; however, variation from one species to another was extreme and easily recognized. They did not study any elasmobranchs. Becker *et al.*,(9) using paper electrophoretic methods, and Drilhon(10) found that gamma globulins were diminished or absent in the sera of teleosts.

Jakowska(11) found a few doubtful plasma cells in teleosts. No observations were found in the literature on the occurrence of plasma

| | Plasma cells (spleen) |
|---|---|
| *Echeneis naucrates*, shark sucker | ± |
| *Lophius piscatorius*, goosefish | ± |
| *Prionotus carolinus*, sea robin | |
| *Seriola zonata*, rudder fish | |
| *Tautoga onitis*, tautog | ++ |
| *Urophycis shuss*, squirrel hake | |
| *Pomatomus saltatrix*, blue fish | |
| *Pseudopleuronectes americanus*, flounder | + |
| *Paralichthys oblongus*, fluke | |
| *Sarda sarda*, bonita | +++ |
| *Palinurichthys perciformis*, barrelfish | |
| *Stenotomus versicolor*, scup | |
| *Homo sapiens*, man | |

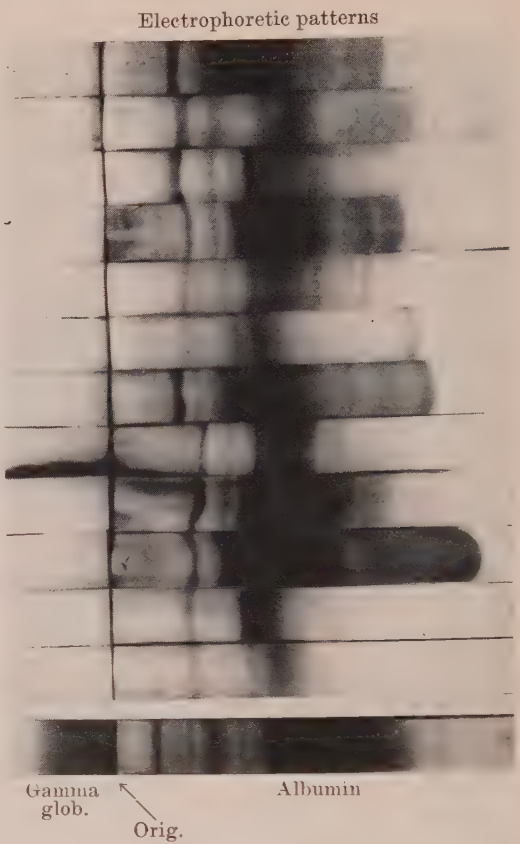


FIG. 3. Starch gel electrophoretic patterns of sera from some marine teleosts. Position at which samples were applied is indicated by arrow below figure. Cathodic area is left of the origin and anodic area is toward the right.

cells in elasmobranchs. Plasma cells have been found in the lymphoid tissues of the ganoid fish, *Polyodon spathula*(12) and in the spleen of the lung fish, *Protopterus ethiopicus*(13).

The presence of plasma cells and gamma globulins in the elasmobranchs and the paucity of plasma cells and absence of gamma globulins in the teleosts as found in the studies reported here suggests that plasma cells may be related to gamma globulins.

It is possible that in teleosts proteins serving the functions of antibodies do not migrate electrophoretically like mammalian gamma globulins. The electrophoretic pattern of each species was complex and some of the bands in the anodic region of the gel might function as antibodies. It is true that in human beings proteins, with the immunologic properties of antibodies, migrate electrophoretically as beta or gamma globulins(14). Attempts should be made to hyperimmunize fish and to follow the response of the serum proteins and plasma cells.

*Summary.* 1) Serum proteins of 18 species of fish have been analyzed by means of starch gel zone electrophoresis. In 10 of these species, observations were also made on occurrence of plasma cells in spleens. 2) Elasmobranchs had typical plasma cells in their spleens and proteins in their serum migrating like gamma globulins upon electrophoresis. Teleosts, however, generally had very few plasma cells in their spleens and no demonstrable gamma globulin in the serum. These observations suggest that among marine fish, there may be an association between plasma cells and gamma globulins.

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## Origin and Fate of Cells in the Medulla of Rat Thymus.* (24226)

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Recently, counts were made of the resting and dividing nuclei of the 4 main cell types present in the cortex of rat thymus (reticular cells, large, medium, and small lymphocytes) and a scheme based on these counts was proposed to explain the production of lymphocytes in this tissue (Fig. 1). Briefly, mitoses of reticular cells would yield an equal number of large lymphocytes and of new reticular cells. The large lymphocytes would then begin a series of successive divisions, so that a total of 8 generations of smaller and smaller lymphocytes would be produced and thus, each initial large lymphocyte would yield 128 "mature" small lymphocytes(1). The present work consists of counting cells and mitoses in the medulla of thymic lobules, using procedures devised to study the cortex(1). The immediate aim was to find out whether or not the pattern of lymphocyte formation observed in the cortex also applied to the medulla. Furthermore, since the investigation of the cortex had given no clues as to how lymphocytes leave the thymus, it was hoped that examination of the medulla would clarify this problem.

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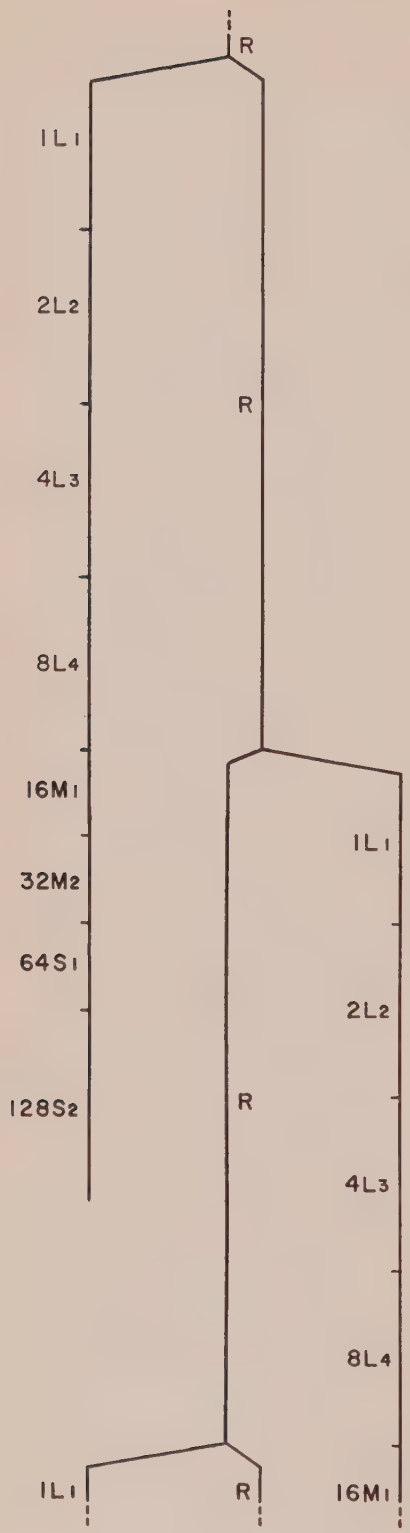
*Methods.* The same thymus sections as were used in investigating the cortex of young adult rats (Bouin-Hollande fixation, Dominici staining) were utilized in the present study. Resting and dividing cells were counted in 625 $\mu^2$-fields of medulla. It had been previously found that the cortex could be divided into 2 layers: peripheral cortex making up 9%, and deep cortex making up 66% of the parenchymal thymus(1). The medulla accounts for the remaining 25%.

*Results. Cytological observations.* The 4 cell types seen in the cortex (Fig. 2) were also present in the medulla, but with a few minor differences in their appearance. The cytoplasm of *reticular cells* stained more intensely and was more distinct in medulla (Fig. 7) than in cortex. The nuclei of some cells contained granules of chromatin-like material often present within vacuoles (Fig. 7, lower center)—a picture interpreted as degeneration(2). Moreover, other stages of degeneration up to complete dissolution of nuclear material were seen, particularly in some reticular cells associated with, or making up Hassall's corpuscles (Fig. 7, upper left).

A few of the *large* and *medium* lymphocytes in medulla had irregularly shaped nuclei. Such irregularities were most pronounced in

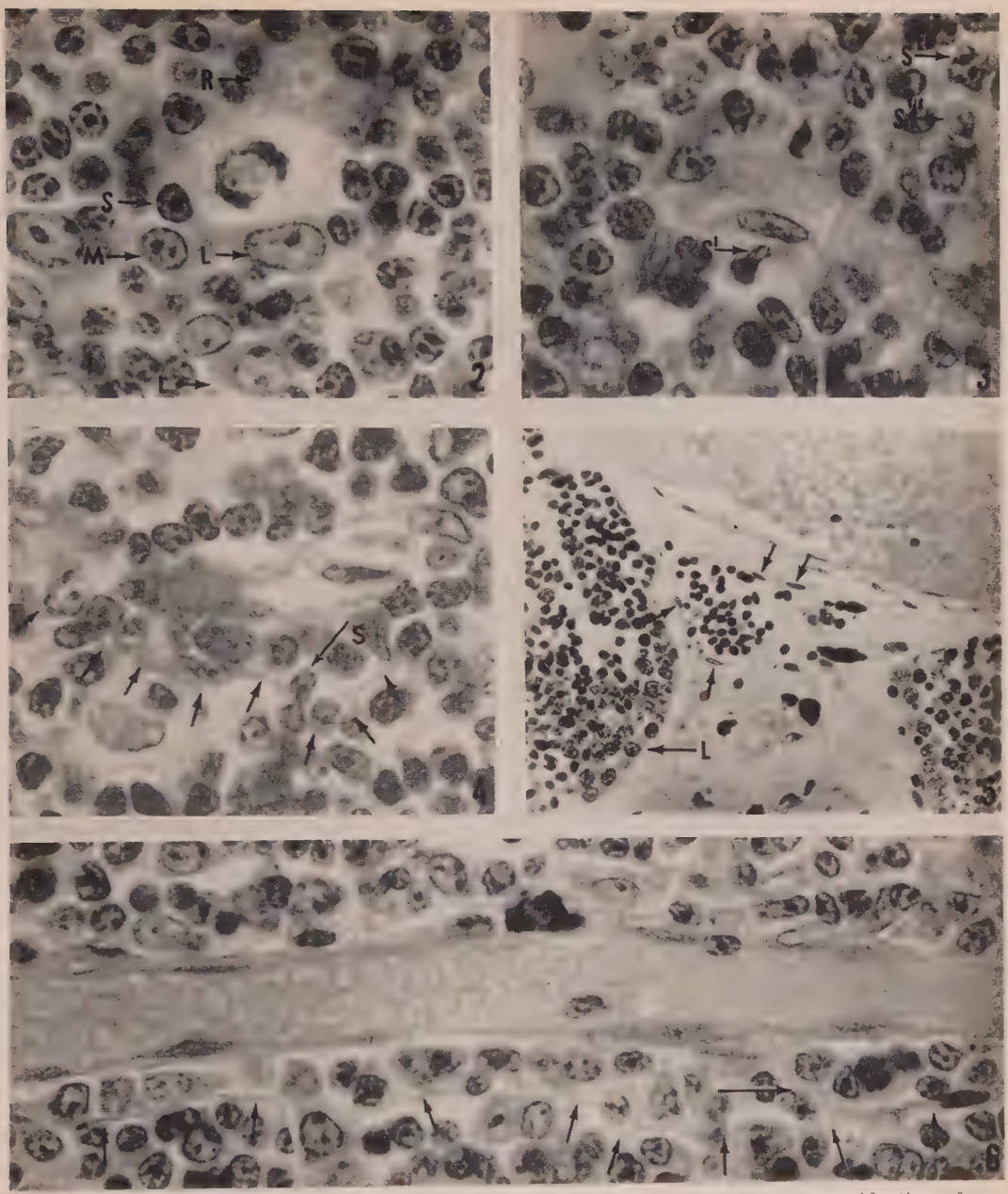



*small* lymphocytes, which in addition had slightly larger and paler nuclei than in cortex(2,4,5). Pseudopod-like processes (Fig. 3, S′ and S″) and tube-like extensions of the nuclei(3; Fig. 3, S‴), rod-like and hour-glass shaped nuclei (Fig. 4, S) and other nuclear distortions were common in the small lymphocytes of medulla, but were rarely seen in cortex. Such figures were interpreted as indicating that the ameboid activity of the cells is pronounced in medulla.

The *vascular system* was examined in thymus sections in the hope of finding clues as to mode of release of lymphocytes(2,6-9). Typical lymph vessels were rare (Fig. 5), but lymphatic spaces associated with venules or other blood vessels were common in medulla and trabeculae. In cross section, such spaces were seen to surround blood vessels, which were thus enclosed as in a coaxial tube. Hence they will be referred to as "perivascular lymphatic channels." These structures, which were seen by Smith(10,11) (although this author may not have appreciated their significance) were composed of thin, barely visible, endothelium-lined walls, which may be collapsed or enclose rows of lymphocytes (Fig. 6). The walls of these perivascular lymphatic channels often showed cells with constricted nuclei undergoing diapedesis (Fig. 4). Similar observations were made in the walls of the enclosed blood vessels (Fig. 8). The cells in diapedesis usually were small lymphocytes (Figs. 4, 8), occasionally other lymphocytes, but never reticular cells. Diapedesis was not observed in cortex.

*Cell counts.* The numbers of cells and mitoses counted per field in the medulla (Table I, right) are presented next to those previ-

FIG. 1. Stem Cell Renewal Pattern of Lymphocyte Formation. Letters R, L, M and S refer to reticular cells, large, medium and small lymphocytes respectively. Each lymphocyte generation is indicated by such a letter preceded by a number indicating how many cells are involved and followed by a subscript referring to generation under consideration. Small horizontal bars symbolize mitoses. Vertical dimension is time. The diagram indicates that at regular intervals, each reticular cell yields on the average another reticular cell and a large lymphocyte, which passes through 4 generations and then produces medium lymphocytes (2 generations), which in turn give rise to small lymphocytes (2 generations).




Thymus of young adult rats stained with the Dominici technic. Figures at a magnification of × 1450, except Fig. 5, × 260.

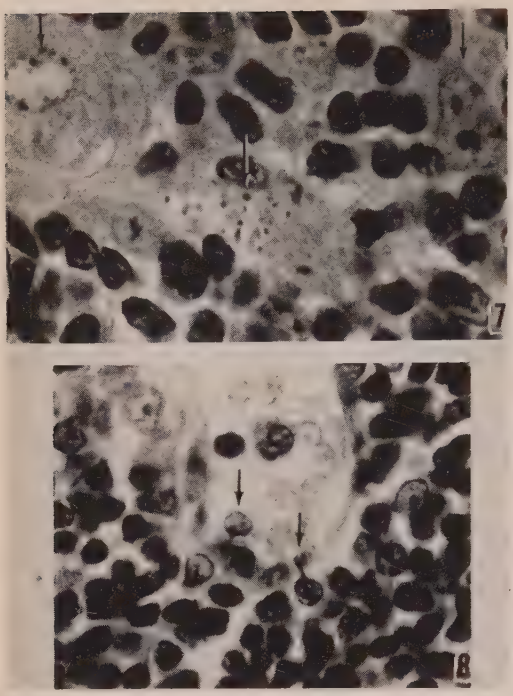
FIG. 2. Cortex. Anaphase of reticular cell may be seen in center. Above, an interphase nucleus of reticular cell (R). Below, arrows point to large lymphocytes (L), a medium (M) and a small (S) lymphocyte.

FIG. 3. Medulla. S′ arrows point to small lymphocyte with its nucleus sending off a large pseudopod-like process. S″ arrow shows crenated nucleus of small lymphocyte. S‴ shows small lymphocyte nucleus sending off a long process in the shape of squirrel tail.

FIG. 4. Medulla. Small arrows line part of wall of a ''perivascular lymphatic channel'' surrounding blood vessel seen above. An hour-glass shaped small lymphocyte nucleus (S) is seen going through this wall.

FIG. 5. Connective tissue trabecula. Small arrows point to endothelial nuclei of wall of typical lymph vessel running close to vein (above). Lymph vessel contains many small and one medium lymphocyte. In nearby cortex the ''L'' arrow shows an island of large lymphocytes.

FIG. 6. Photomontage of vein and associated ''perivascular lymphatic channel'' in medulla. In lower portion of Figure, a series of vertical arrows point to wall of perivascular lymphatic channel. One attachment to wall of vein is shown by horizontal arrow at lower right. In upper portion of Figure, the wall of lymphatic channel stands out on right hand side, but is collapsed and barely visible on the left.



Thymus of young adult rats stained with the Dominici technic. × 1450.

FIG. 7. Reticular cells in medulla. Upper right arrow points to nucleus of healthy reticular cell. Upper left arrow shows degenerating nucleus of reticular cell within Hassall's corpuscle. Note clumping of chromatin material. Central arrow shows nucleus with similar clumping in an isolated reticular cell.

FIG. 8. Two small lymphocytes undergoing diapedesis through walls of vein located at limit between cortex and medulla. On right, small lymphocyte is outside the vein, and sends off into the wall a process with claw-like arrangement of chromatin. On left, small lymphocyte is within vein, but a nuclear process (not quite in focus) is held within the wall.

ously reported for the cortex (Table I, left). The medulla contained nearly 4 times more *reticular cells,* but 25 times less *large lymphocytes* and 10 times less *medium lymphocytes* per field than the cortex. The numbers of mitoses varied in the same direction, so that mitotic indices for each of these 3 cell types were comparable in both regions. (No standard errors are given for the numbers of large lymphocytes and their mitoses, since these cells were rare in the medulla, where only 32 were counted.) The number of *small lymphocytes* in medulla was 1.7 times less than in cortex, while the number of mitoses was decreased by a factor of 4, so that the mitotic index of these cells was lower than in cortex.

*Discussion.* Except for small lymphocytes, mitotic indices were comparable in cortex and medulla (Table I); and therefore, reticular cells, as well as large and medium lymphocytes, have a comparable life span in the 2 regions(1). Furthermore, mitoses were abundant enough to indicate a continuous renewal of the 4 cell types(1). Thus, it was felt likely that lymphocytes evolve in a similar manner in cortex and medulla. To test this hypothesis, the relation of each cell type to its precursor was examined in detail in the medulla. Whenever the relation differed from that observed in cortex, an explanation was sought in the cytological and architectural features characteristic of the medulla.

*Relation of reticular cells to large lymphocytes.* The number of large lymphocytes in medulla was only .025 per field. If reticular cells yielded large lymphocytes at the same rate as in cortex, this number would be 2.61 per field (Table II), *i.e.* about 100 times more. Thus, only about 1% of reticular cell mitoses may produce large lymphocytes, the other 99% yielding new reticular cells instead.

The production of reticular cells as a result of mitotic activity must be balanced by an equivalent loss to maintain a steady state.

TABLE I. Number of Cells and Mitoses per Field, and Mitotic Indices in Cortex and Medulla of the Thymus of 10-Week-Old Male Rats.

| | Total cortex* | | | Medulla | | |
|---|---|---|---|---|---|---|
| | No. of cells | No. of mitoses | Mitotic index | No. of cells | No. of mitoses | Mitotic index |
| Reticular cells | .20 ± .03† | .003 ± .001† | .015 | .798 ± .06† | .007 ± .003† | .009‡ |
| Large lymphocytes | .66 ± .08 | .046 ± .004 | .065 | .025 | .0014 | .053 |
| Medium " | 1.15 ± .05 | .172 ± .011 | .131 | .114 ± .04 | .019 ± .002 | .143 |
| Small " | 15.75 ± .39 | .240 ± .011 | .015 | 9.040 ± .40 | .065 ± .008 | .007 |

* From reference 1. † ± S.E. ‡ A recount of 1000 reticular cells in medulla yielded a mitotic index of .010.

TABLE II. Numbers of Cells Found in Medulla of Thymus of 10-Week-Old Male Rats as Compared with Numbers Expected from Population of Immediate Precursor.

| | Experimental | Expected | Difference |
|---|---|---|---|
| Reticular cells | .790 | | |
| Large lymphocytes | .025 | 2.61 | −2.56 |
| Medium " | .114 | .045 | + .069 |
| Small " | 9.04 | .840 | +8.20 |

Indeed, the signs of nuclear degeneration up to complete disintegration found in some reticular cells (2; Fig. 7) indicate that at all times some of these cells are dying. Accordingly, Hassall's corpuscles may be considered to be structures into which reticular cells become aggregated and eventually degenerate.

*Relation of medium to large lymphocytes and of small to medium lymphocytes.* In the medulla, comparison of cell counts for medium and small lymphocytes with the values expected from the numbers of their immediate precursors (Table II) revealed that medium lymphocytes were more than twice, and small lymphocytes more than 10 times as numerous as expected. Where do the extra cells come from? It was suggested that lymphocytes migrate from cortex to medulla(2, 4) and then enter the circulation(2,8). Indeed, since diapedesis of lymphocytes occurs in medulla (Fig. 4, 8) but not in cortex, lymphocytes must pass into the medulla before entering blood and lymph vessels. From Table II, it may be calculated that, if about 60% of the medium and 91% of the small lymphocytes present in medulla were to come from cortex, the counts obtained for these 2 cell types would be explained satisfactorily.‡

Examination of the cells undergoing diapedesis and those present within lymph channels revealed that medium lymphocytes and even the odd large lymphocyte may enter the circulation. However, the larger the lymphocyte, the less it migrates from cortex to medulla to circulation. Those medium and large lymphocytes present in blood and lymph

TABLE III. Behavior of Small Lymphocytes of First ($S_1$) and Second ($S_2$) Generation in the 3 Zones of the Thymus of 10-Week-Old Male Rats.

| | Peripheral cortex | Deep cortex | Medulla |
|---|---|---|---|
| Total No. of small lymphocytes* | 128 | 1053 | 226 |
| No. of Type $S_1$† | 23 | 99 | 11 |
| No. of Type $S_2$—Total No.‡ | 105 | 954 | 215 |
| No. arising *in situ* | 105 | 774 | 18 |
| from peripheral cortex | | 180 | 37 |
| from deep cortex§ | 0 | | 160 |
| Mean time spent by $S_2$ cells in each zone§ (using life span of $S_1$ as unity) | 2.25 | 3.19 | .81 |

* Relative numbers obtained as follows: Counts of small lymphocytes/field for the 3 regions were multiplied respectively by 9, 66 and 25 (% of volume of parenchymal thymus which these regions occupy).

† Calculated from No. of small lymphocyte mitoses (all attributed to $S_1$), assuming that life span of $S_1$ in any region is 2.25 times as small as the mean time spent by $S_2$ cells in peripheral cortex(1).

‡ Obtained by difference between the above two figures.

§ Calculated from contributions made to each $S_2$ pool by $S_1$ cells from the various zones, assuming steady state conditions. (Detail of calculations will be sent on request.)

‡ Number of medium lymphocytes which would migrate under these conditions may be estimated, if it is remembered that the ratio of volumes of medulla to cortex was exactly 1 to 3, i.e., 25 fields of medulla for 75 of cortex. Data on the right in Table II indicate that in each field a mean of 0.069 medium lymphocyte would come from the cortex, that is 1.7 for 25 fields. Number of medium lymphocytes/field in cortex is 1.15 ± .05 (Table I), i.e., 86.25 ± 3.8 for 75 fields. Number of these cells leaving the cortex (1.7) to enter the medulla is therefore relatively small, smaller even than standard error of the number of these cells in cortex (3.8), so that the loss of medium lymphocytes to the medulla is too small to affect the reasoning in reference 1.

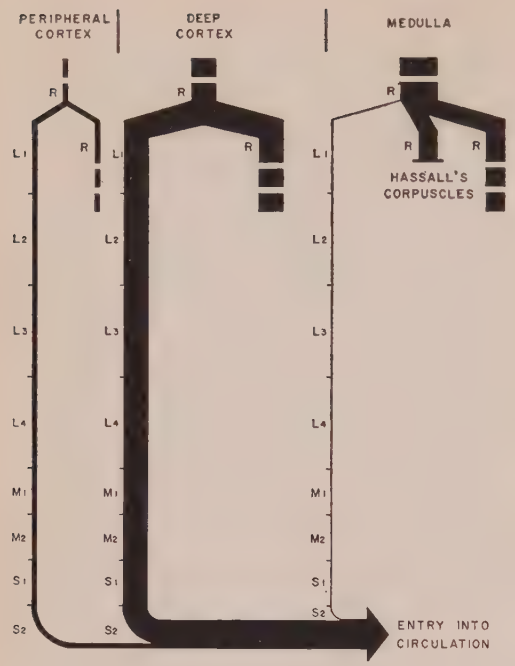



FIG. 9. Formation and migration of lymphocytes in peripheral and deep cortex and in medulla of rat thymus (lettering as in Fig. 1). Basic pattern in Fig. 1 is repeated for each of the 3 zones, with thickness of long vertical portion made proportional to amount of local lymphocyte production. At top of diagram, reticular cells are dividing. In peripheral and deep cortex, each such division yields on the average a large lymphocyte ($L_1$) and another reticular cell (R). In medulla, few large lymphocytes are produced from reticular cells, most divisions yielding reticular cells, of which on the average half die, frequently in Hassall's corpuscles. At base of diagram, the lengths of horizontal portions for the 3 zones were made proportional to mean time spent by $S_2$ cells in each. Vertical line standing for peripheral cortex is curved to the right to join line standing for deep cortex; then both together join line standing for medulla, thus indicating pathway of $S_2$ migration to medulla and hence to circulation.

channels were seen to undergo mitosis occasionally, just as they do in the parenchyma. It appears that their life span is the same not only in cortex and medulla (Table I), but perhaps even in the circulation, as indicated by unpublished studies of "medium" lymphocytes labelled after thymidine-$H^3$ injection (B. J. Bryant and Lola S. Kelly). Thus, medium lymphocytes—and presumably the odd large lymphocyte—entering the circulation would continue their evolution towards the "mature" small lymphocyte stage (Fig. 1).

*Origin and migration of cells in thymus.* An analysis of cell and mitotic counts in cortex had led to the elaboration of a Stem Cell Renewal Pattern of Lymphocyte Formation (1). The same method of analysis did not yield a clearcut pattern for the medulla. This may be explained by the failure of reticular cells to yield large lymphocytes as in cortex, and also by the complication introduced by migration of medium and small lymphocytes, which are thus added to the cells formed in the medulla.

Our tentative conclusions regarding the role of cortex and medulla in formation and migration of lymphocytes are illustrated in a diagram (Fig. 9), which was constructed as if only second generation small lymphocytes ($S_2$) were involved in migration (Table III). This assumption was adopted for the sake of simplicity, since it was known that small lymphocytes play a major role in migratory processes, and precise data concerning the part played by other lymphocytes were lacking.

The *peripheral cortex* (Fig. 9, left) shows the typical pattern pictured in Fig. 1, but with the base of the diagram curved to the right to indicate that small lymphocytes ($S_2$) arising in this zone migrate across the deep cortex where they join with more $S_2$ cells and together migrate towards and into the medulla.

Again, lymphocytes are formed in *deep cortex* according to the basic pattern but in greater numbers than in peripheral cortex, as indicated by the thicker line for the central diagram in Fig. 9. That small lymphocytes spend more time in deep than in peripheral cortex (Table III, last line), is represented in the diagram by the greater length of the horizontal portion of the diagram for deep than for peripheral cortex.

In *medulla,* the pattern is changed as far as reticular cells are concerned, since the mitoses of these cells yield reticular cells almost exclusively, instead of large lymphocytes as in cortex; and the new reticular cells either divide again or die by degeneration, an occurrence observed in scattered cells as well as in some of those grouped in Hassall's cor-

puscles. The various types of lymphocytes seem to reproduce in the same manner as in cortex, but the sizable population of small lymphocytes found in medulla comes largely from cortex. The concept of the medulla as a passageway for these cells is consistent with the observations of ameboid activity and of diapedesis into the vessels of this region (Figs. 4 and 8). Lymphocytes which have entered the perivascular lymphatic channels (Fig. 6) may reach the main circulation in 2 ways: 1) by diapedesis from these channels into the enclosed blood vessels and 2) by travelling along these channels to the main lymphatic circulation.

In conclusion, it seems that an important function of the thymus is to produce lymphocytes(12,13) to be delivered to the circulation through the medulla.

*Summary.* 1) Counts of resting and dividing cells in the thymic medulla of 10-week-old male rats revealed that reticular cells are more abundant than in cortex, but the 3 types of lymphocytes (large, medium, small) are less abundant than in cortex. However, except for small lymphocytes, each cell type has approximately the same mitotic index and, therefore, approximately the same life span in the 2 regions. 2) In view of the small number of large lymphocytes present, it appears that the evolution of reticular cells into lymphocytes is the exception in medulla, instead of being the rule as in cortex. Mitoses of reticular cells would mainly give rise to other reticular cells. Since these cells frequently show signs of degeneration, it is suggested that their loss through degeneration balances the overproduction indicated by their high mitotic activity. Clumps of reticular cells, many of which are in various stages of degeneration, make up the structures known as Hassall's corpuscles. 3) That the small lymphocyte is the end product of a series of 8 successive generations from large lymphocytes through smaller and smaller cells, seems to hold in medulla as in cortex. However, the presence of many more small lymphocytes in medulla than expected from the number of medium lymphocytes raised the possibility that small lymphocytes immigrate from cortex. Indeed, since diapedesis of small lymphocytes was commonly seen across the walls of the perivascular lymphatic channels and blood vessels in medulla but not in cortex, a migration of cortex-formed small lymphocytes through the medulla must be postulated to allow them to reach the circulation. To a lesser extent, medium lymphocytes (and perhaps even the odd large lymphocyte) may also migrate from cortex to medulla and from there to the circulation. It is concluded that only a small degree of lymphocyte formation occurs in medulla. This region is mainly a passageway allowing the lymphocytes arising in cortex to reach the circulation.

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